University of Alberta

Characterization of Carbamoyl Phosphate Synthetase 1 palmitoylation and development of a chemical biology approach to detect and identify new palmitoylated proteins

by

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Abstract

S-acylation is the modification of proteins by a variety of fatty acids on cysteine residues via thioester bond. Since palmitate (C16:0) is the most abundant fatty acid in cells and as such the most frequently found attached to proteins, S-acylation is preferentially referred to as palmitoylation. In mitochondria, palmitoylation occurs mainly in the matrix where metabolic processes take place, suggesting that palmitate plays a role in the regulation of metabolism. Rat liver carbamoyl phosphate synthetase 1 (CPS 1) is a prominent palmitoylated 165 kDa mitochondrial protein. Palmitoylation inhibits CPS 1 activity at physiological concentrations of palmitoyl-CoA. This inhibition corresponds to an irreversible inactivation of CPS 1 and occurred in a time- and concentration-dependent manner. Palmitoylation of CPS 1 was prevented by pre-incubation with *N*-ethylmaleimide and 5'-*p*-fluorosulfonylbenzoyladenosine, an ATP analog that reacts with CPS 1 active site cysteine residues, suggesting that palmitoylation of CPS 1 very likely occurs on at least one of its essential active site cysteine residues. Site-directed mutagenesis analysis of these cysteines failed to confirm the palmitoylation site on CPS 1 due to a lack of an appropriate recombinant expression system. In order to facilitate and promote palmitoylation research, we developed a non-radioactive method allowing for the rapid detection and identification of mitochondrial palmitoylated proteins. This method is based on

the modified Staudinger reaction in which an azido-palmitate moiety is incorporated into proteins and specifically reacted with a tagged-triaryl-phosphine capture reagent. The resulting modified proteins can be detected using western blot/ECL. Our results show that mitochondrial proteins are azido-fatty acylated on cysteine residues, suggesting that the azido moiety does not affect the specificity of the modification. This methodology allowed the identification of pyruvate carboxylase (PC) and aspartate aminotransferase (AAT) as putative mitochondrial palmitoylated proteins.

Regulation of metabolic enzymes such as CPS 1, PC and AAT by long-chain fatty acyl-CoAs could reduce amino acid degradation and urea genesis, thereby contributing to nitrogen sparing during prolonged starvation. These findings may be relevant in cases of long-chain acyl-CoA dehydrogenase deficiency, obesity and diabetes where cellular fatty acyl-CoA concentrations are established to be higher than normal.

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CHAPTER 1

INTRODUCTION

1.1 COVALENT MODIFICATIONS OF PROTEINS

Posttranslational protein modifications are important for protein transport, cellular localization and regulation of enzymatic activity. Protein lipidation, the covalent modification of proteins by lipids, alters their physical and functional properties providing modulation of protein-protein interactions, regulation of enzymatic activity, stabilization of protein structure and granting hydrophobicity to allow association with cellular membranes.

Protein lipidation is divided into four major categories (Table 1.1): prenylation, glypiation, cholesteroylation and fatty acylation (Casey, 1995; Porter, *et al.*, 1996; Bhatnagar and Gordon, 1997). Prenylation is the covalent modification of proteins by the isoprenoids farnesyl (C15:0) and geranylgeranyl (C20:0). These lipids are attached posttranslationally to one or more cysteine residues at or near the C-terminus of the protein via a stable thioether bond. Glypiation is the covalent modification of proteins at their C-terminus by glycosylphosphatidylinositol structures. Cholesteroylation is the covalent modification of proteins by cholesterol (Porter, *et al.*, 1996). Finally, protein fatty acylation is the covalent modification of proteins by fatty acids and is divided into two categories: myristoylation and palmitoylation (Bhatnagar and Gordon, 1997).

Historically, fatty acylation of eukaryotic proteins was divided into two classes: cotranslational addition of myristate to N-terminal glycine through amide linkage (myristoylation) and posttranslational addition of palmitate

Table 1.1 Characteristics of the major types of lipidated proteins.

The four major classes of lipidation and their subclasses. The subunit to which the lipid is attached is indicated in parentheses after the trimeric G protein. NRTKs- non receptor tyrosine kinases, retinal GRK-specific receptor kinases (Adapted from Casey, 1995 and Bhatnagar & Gordon, 1997).

Modification	Subclass of lipid attached	Examples	Position of the modification	Type of bond
Prenylation	Farnesyl	Trimeric G proteins (γ), small G proteins, retinal GRK	C-terminus	Thioether; stable
	Geranyl-geranyl			
Glypiation	Complex structure containing ethanolamine, sugars and phosphatidylinositol	Many cell-surface proteins	C-terminus	Amide; stable
Cholesteroylation	Colesterol	Hedgehog family of proteins	C-terminus	Ester; labile
Fatty acylation	N-Myristoyl	Trimeric G proteins (α), NRTKs	N-terminus	Amide; stable
	S-Palmitoyl	Trimeric G proteins (α), Ras, MMSDH	C-terminus, N-terminus, internal	Thioester; Labile

through thioester linkage to cysteine (palmitoylation) (Magee and Courtneidge, 1985). However, fatty acylation of eukaryotic proteins is more diverse, and this categorization has been expanded to include modification of other amino acid residues with amide- and ester-linked fatty acids. For example, ghrelin, a growth-hormone-releasing peptide from the stomach, is modified by octanoate through an oxyester bond to a serine residue (Kojima, *et al.*, 1999). Moreover, components of spider venoms are O-palmitoylated on a threonine residue (Branton, *et al.*, 1993), and the secreted signaling protein Sonic Hedgehog is palmitoylated through an amide bond (Pepinsky, *et al.*, 1998). Fatty acids linked by amide bonds are found in the bacterial toxin hemolysin (Stanley, *et al.*, 1998), tumor necrosis factor (Stevenson, *et al.*, 1992) and interleukin 1α precursor (Stevenson, *et al.*, 1993).

Traditionally, myristoylation was defined as a cotranslational process. In *N*-myristoylation, the fourteen-carbon fatty acid myristate is added to an essential N-terminal glycine residue via an amide bond after the removal of the initiator methionine residue. The consensus sequence recognized by *N*-myristoyl-transferase (NMT) is M-G-X-X-X-S/T/C with preferentially a lysine or arginine residue at position 7 and/or 8 (Farazi, *et al.*, 2001; Resh, 2004). By itself, a myristoyl moiety added to a protein is not sufficient to confer stable membrane anchoring. Instead, an adjacent or remote second signal in the form of a polybasic stretch of amino acids or of one or two palmitoyl-cysteine residues is

often required for stable membrane binding and proper membrane targeting (McCabe and Berthiaume, 1999; Resh, 2004).

Following the original demonstration of posttranslational myristoylation of truncated Bid (myr-t-Bid) (Zha, *et al.*, 2000), posttranslational myristoylation of two caspase-cleaved cytoskeletal proteins, actin and gelsolin, was demonstrated (Utsumi, *et al.*, 2003) as well as the posttranslational myristoylation of the serine/threonine kinase p21-activated kinase 2 (Vilas, *et al.*, 2006).

1.2 PALMITOYLATION

Palmitoylation is defined as the posttranslational addition of palmitate (C16:0) to cysteine residues via a thioester linkage (Linder and Deschenes, 2003) (Table 1.2). Many aspects of protein palmitoylation have thoroughly been reviewed by Bijlmakers and Marsh (2003), Smotrys and Linder (2004) and Magee and Seabra (2005).

For protein palmitoylation to occur, palmitate must be activated in the form of its coenzyme A derivative, palmitoyl-CoA (Berger and Schmidt, 1984; Bizzozero and Lees, 1986). Palmitoylation is a reversible modification (Mumby, *et al.*, 1994) that has been recently shown to regulate the subcellular localization of several proteins involved in signal transduction processes (Casey, 1995; Milligan, *et al.*, 1995; Dunphy and Linder, 1998). The labile thioester linkage permits repeated cycles of palmitoylation and

Table 1.2 Characteristics of the two categories of fatty acylation: myristoylation and palmitoylation.

NMT: *N*- myristoyltransferase; PAT: protein acyl transferase

Characteristic	N-Myristoylation	S-Palmitoylation
Fatty acid	Myristic (C 14:0)	Palmitic (C 16:0)
Type of linkage	Amide	Thioester
Modified residue	Glycine	Cysteine
Reversibility	Irreversible	Reversible
Enzymology	NMT	PAT and autoacylation
Consensus sequence	MGXXX(C/S/T)XX	None apparent

depalmitoylation of a protein and, as such, can act as a molecular switch for a protein during its existence in a cell (James and Olson, 1990).

Many studies have shown that not only palmitate (C16:0) but also palmitoleate (C16:1*n*-9), stearate (C18:0), and oleate (C18:1*n*-9) can be found attached to proteins (Berger and Schmidt, 1984; Bizzozero and Lees, 1986; Casey, 1995; Veit, *et al.*, 1996). Thus, S-acylation or thioacylation is a more general and accurate term used to describe this posttranslational protein modification.

1.2.1 Structural requirements for protein palmitoylation

In contrast to N-myristoylation, palmitoylation does not have a well defined consensus sequence other than a prerequisite for cysteine (Smotrys and Linder, 2004). However, several studies characterizing different palmitoylated proteins have unveiled some sequences contexts that serve as acceptor sites for palmitoylation. Examples of these palmitoylation motifs are illustrated in Table 1.3.

Palmitoylation is found both on integral and peripheral membrane proteins. Cellular integral membrane proteins are palmitoylated on cysteine residues that are either close to the transmembrane/ cytoplasmic domain boundary, or located in their cytoplasmic domain (Bouvier, *et al.*, 1995; Ponimaskin and Schmidt, 1995). In some cases the transmembrane domain can influence palmitoylation. Non-hydrophobic residues in the transmembrane domain can

Table 1.3 Palmitoylation motifs.

Palmitoylation sequence contexts recently identified and examples of each of them (Modified from Smotrys & Linder 2003). Palmitoylated cysteines are in red; the superscript numbers refer to the amino acid position in the protein sequence. Abbreviations used are farn, farnesyl isoprenoid; gg, geranylgeranyl isoprenoid.

Palmitoylation motifs	Examples	Sequence
Prenylation and palmitoylation	H-Ras	-GCMSCKCfarn
	N-Ras	-GCMGLPCfarn
	R-Ras	-KGGGCPCgg
	Rho B	-QNGCINCCfarn/gg
	TC 10	-GSRCINCCfarn
N-myristoylation and palmitoylation	$p59^{fyn}$	myr-GCVQCKDKE-
	$p56^{lck}$	myr-GCGCSSHPE-
	$G_{i\alpha1}$	myr-GCTLSAEDK-
N-terminal motifs	GAP-43	^{1}MLCCM-
	PSD-95	^{1}MDCLCITT-
C-terminal motif	Yck2 (*S. cerevisiae*)	-FFSKLGCC
	AtRac8 (*A. thaliana*)	-GCLSNILCGKN
Cysteine string motifs	SNAP-25b	^{83}KFCGLCVCPCNKL^{95}

favor palmitoylation of integral membrane proteins (Ponimaskin and Schmidt, 1998). Both the length and composition of the transmembrane domain and the length of the cytoplasmic tail appear to influence the choice of fatty acid for acylation. Proteins with short basic tails are modified with stearate whereas those with longer tails are primarily palmitoylated (Ponimaskin and Schmidt, 1995; Veit, *et al.*, 1996; Ponimaskin and Schmidt, 1998).

For peripheral membrane proteins, palmitate is found attached either close to myristic acid or prenyl groups, or in the absence of other acylations.

Members of the Ras superfamily are prenylated at their C-termini and several, including H-Ras and N-Ras, are also palmitoylated (Hancock, 2003). Palmitoylation of N-Ras and H-Ras determines Golgi targeting and transport to the plasma membrane (Hancock, 2003)(Table 1.3). Other proteins of the Ras family such as R-Ras, Rho B, TC 21, TC 10, and Rap 2b are predicted to be palmitoylated and geranylgeranylated based on their amino acid sequence (Michaelson, *et al.*, 2001; Furuhjelm and Peranen, 2003).

Proteins that are both N-myristoylated and palmitoylated have the palmitoylated modified cysteine residue in close proximity to the N-myristoylated glycine (Dunphy and Linder, 1998; Resh, 1999; Navarro-Lerida, *et al.*, 2002). A typical example of this motif includes all members of the Src family of protein tyrosine kinases and the $G_{i\alpha}$ subfamily (Table 1.3). Newly synthesized Src family kinases and G_{α} subunits are cotranslationally myristoylated, but they do not stably associate with membranes until

palmitoylation has occured (Alland, *et al.*, 1994). In most cases, the amino acid residues surrounding the palmitoylated cysteine are not important when N-myristoylation concomitantly takes place (Schroeder, *et al.*, 1996; Navarro-Lerida, *et al.*, 2002).

When palmitoylation is the only modification that takes place on a protein, the modified cysteine residue(s) is/are located within the first 25 amino acids. In this case, there is no apparent palmitoylation motif exept for the presence of cysteines. A classical example of a protein exclusively palmitoylated is PSD-95 (postsynaptic density protein of 95 kDa) (El-Husseini, *et al.*, 2000). PSD-95 is a scaffolding protein that is important for clustering neuronal receptors at postsynaptic densities. Palmitoylation of PSD-95 occurs at cysteine residues at positions 3 and 5. The first 13 amino acids are sufficient for palmitoylation to take place.

SNAP-25 (synaptosome-associated protein 25 kDa), a neuronal SNARE protein found at the plasma membrane that mediates synaptic vesicle exocytosis, is found to be palmitoylated on several cysteine residues clustered in a short amino acid sequence. Mutation of two of SNAP-25's four cysteine residues results in a complete loss of palmitoylation, thus demonstrating a requirement for at least three of the cysteine residues for incorporation of radiolabeled palmitate to occur (Lane and Liu, 1997). The requirement for three cysteine residues on SNAP-25 is of interest, since it suggests that a

distinct mechanism (or protein acyltransferase) may be operating for proteins with cysteine clusters.

AtRac8 offers an interesting case. AtRac8 is a Rho GTPase from plants that was found to be palmitoylated at its C-terminus. However, the modification takes place on a canonical prenylation site (Lavy, *et al.*, 2002). This example illustrates that palmitoylation sequence motifs cannot always be used as predictors of the lipidation state of a protein.

1.2.2 Mechanisms of protein palmitoylation

It has been proposed that palmitoylation occurs either spontaneously (autocylation) or catalyzed by an enzyme (protein acyltransferase) (Berthiaume, *et al.*, 1994; Corvi, *et al.*, 2001; Lobo, *et al.*, 2002; Roth, *et al.*, 2002). The understanding of the enzymatic action responsible for protein palmitoylation is still developing due to the lack of well characterized protein acyltransferases (PATs). Besides, the existence of such proteins has long been questioned, since biochemical studies which were successful in enriching PAT activity failed to identify the respective protein (Berthiaume and Resh, 1995; Dunphy, *et al.*, 1996; Veit, *et al.*, 1996; Veit, *et al.*, 1998; Linder and Deschenes, 2003). Furthermore, palmitoylated proteins did not appear to have a clear consensus sequence for the potential enzyme to recognize the target cysteine residue susceptible to be modified. And finally, proteins containing a target cysteine residue can be autoacylated *in vitro* at the same sites that are found *in vivo*. Despite these considerations, in the past four

years there have been important developments in this field and three yeast proteins that promote palmitoylation have been described: Erf2/Erf4, Akr1 and Pfa3. These enzymes palmitoylate intracellular proteins. Erf2/Erf4 (effect on ras function) complex stimulates palmitoylation of Ras2 (yeast homologue to H-Ras and N-Ras) substrates, Akr1 (ankiryn-repeat-containing protein) palmitoylates Yck2 (yeast casein kinase 2) and Pfa3 (protein fatty acyltransferase 3) has been described as PAT for Vac8 (Lobo, *et al.*, 2002; Roth, *et al.*, 2002; Smotrys, *et al.*, 2005).

Deschenes and collegues identified Erf2 and Erf4 as necessary for the targeting of Ras2 to membranes (Bartels, *et al.*, 1999). Both proteins seemed to be involved in Ras2 palmitoylation, since this modification was suppressed in erf2Δ and erf4Δ mutants (Bartels, *et al.*, 1999). Erf2 is a transmembrane protein that recruits Erf4 to the endoplasmic reticulum (Zhao, *et al.*, 2002). A weak hydrophobic cluster present in the C-terminal half of the protein is essential for Erf4 interaction with Erf2 (Zhao, *et al.*, 2002). Erf2 contains a cysteine-rich domain (CRD), including the typical asp-his-his-cys sequence (DHHC box)(Putilina, *et al.*, 1999), and mutations in this sequence inhibit the *in vitro* palmitoylation activity of the Erf2/4 complex and interfere with Ras2 palmitoylation *in vivo* (Lobo, *et al.*, 2002).

Akr1 was shown to palmitoylate Yck2 at the Golgi (Roth, 2002). Similar to Erf2, Akr1 contains a DHHC-CRD box (Putilina, *et al.*, 1999). Purified Akr1 promoted palmitoylation of both Yck2 and itself and this activity was lost on

mutation of the DHHC box of Akr1 (Roth, *et al.*, 2002). Akr1, like Erf2, has several predicted transmembrane domains and behaves as an integral membrane protein (Bartels, *et al.*, 1999; Roth, *et al.*, 2002).

Pfa3 was linked to Vac8 palmitoylation based on its role in the vacuolar fusion process (Smotrys, *et al.*, 2005) and because it is required for efficient targeting of Vac8 to the vacuole membrane (Hou, *et al.*, 2005). Pfa3 was also shown to contain a classical DHHC-CRD (Smotrys, *et al.*, 2005).

The common element shared by Akr1, Erf2 and Pfa3 is the DHHC-CRD, a protein module that is a variation of cysteine-rich zinc-finger domains (Bohm, *et al.*, 1997; Putilina, *et al.*, 1999). The DHHC-CRD is hypothesized to be a palmitoyltransferase domain (Lobo, *et al.*, 2002; Roth, *et al.*, 2002) that is directly involved in palmitoylation (Lobo, *et al.*, 2002; Roth, *et al.*, 2002).

The initial identification of Erf2 and Akr1 as acyltransferases and the fact that both proteins share a DHHC-CRD led to a screening to identify genes coding for proteins containing this motif. Using this consensus sequence, more than 120 DHHC-CRD genes have been found in the databases, with more than 20 in the human genome (Linder and Deschenes, 2004). Some cloned mammalian proteins that contain a DHHC-CRD include: huntingtin interacting protein 14 (HIP14) (Singaraja, *et al.*, 2002), the Golgi-specific DHHC zinc-finger protein (Uemura, *et al.*, 2002), the proapoptotic c-Abl-interacting protein named Aph2 (Li, *et al.*, 2002), and the protein named "Sertoli cell gene" with a zinc-finger domain (SERZ) (Chaudhary and Skinner, 2002). In 2004 HIP14

was shown to have PATactivity towards neuronal proteins, including SNAP-25, PSD-95, GAD65, synaptotagmin I, and huntingtin (Huang, *et al.*, 2004). However, the PAT activity of the remaining cloned mammalian proteins containing a DHHC-CRD mentioned above remains to be tested.

PATs do not show to be highly specific towards the lipid moiety with palmitate and oleate being the preferred substrates over myristate and shorter length CoA derivatives (Lobo, *et al.*, 2002).

Another class of palmitoyltransferases that palmitoylate secreted proteins has been described. These palmitoyltransferases modify important morphogenic signaling molecules such as Hedgehog and Wnt. Skinny hedgehog is required for the palmitoylation of Hedgehog (Chamoun, *et al.*, 2001), while Porcupine is required for the modification of Wnt proteins (Zhai, *et al.*, 2004). Both Skinny hedgehog and Porcupine have sequence homology to the membrane bound O-acyltransferases (MBOATs), but lack the typical DHHC-CRD found in PATs (Hofmann, 2000).

As mentioned before, it has been reported that acylation of peptides and proteins can occur *in vitro* in the absence of an enzyme using palmitoyl-CoA as the donor. Autoacylation of purified proteins occurs at the same specific site as enzyme-mediated reaction *in vivo*, although at a slower rate (Leventis, *et al.*, 1997; Bano, *et al.*, 1998; Veit, *et al.*, 1998). Bizzozero and collegues noted that under appropriate conditions, the activation energy required to transfer palmitate from palmitoyl-CoA to a peptide is one-fifth of the energy that is

required for enzyme-catalyzed acyl-transfer reactions (Bharadwaj and Bizzozero, 1995) and thus, acylation can occur spontaneously. The crucial aspect of this transfer reaction is the formation of a thiolate as a target for palmitate (Bizzozero, *et al.*, 2001). The thiolate anion can then act as a nucleophile on the thioester bond of palmitoyl-CoA to catalyze the generation of the palmitoylated protein. In cells, this process could be regulated at two levels: the rate of the formation of the thiolate anion and the palmitoyl-CoA availability. The amino acids surrounding the cysteine susceptible to be acylated can influence the proximity and orientation of the cysteine to a membrane as well as the ability of that cysteine's thiol group to ionize (Quesnel and Silvius, 1994; Bharadwaj and Bizzozero, 1995; Bano, *et al.*, 1998). In fact, it has been shown that acidic amino acids surrounding a cysteine residue are inhibitors of autoacylation, while basic and hydrophobic amino acids promote autoacylation (Belanger, *et al.*, 2001). It has been demonstrated that the promoting effect on palmitoylation by basic and aromatic amino acids is mediated by a decrease of the pKa of the cysteine (Bizzozero, *et al.*, 2001). All these studies demonstrate that the sequence context surrounding a cysteine residue is important and influences autoacylation. The second level at which spontaneous acylation may be regulated in cells is the palmitoyl-CoA availability. Eukaryotic cells maintain a low free palmitoyl-CoA concentration in the cytosol since most of it is bound to acyl-CoA binding protein (ACBP).

Autoacylation was also shown to play a physiological role in the regulation of several mitochondrial enzymes such as pyruvate dehydrogenase, ADP/ATP translocase, glutamate dehydrogenase, carbamoyl phosphate synthetase 1 and methylmalonate semialdehyde dehydrogenase (Morel, *et al.*, 1974; Kawaguchi and Bloch, 1976; Moore, *et al.*, 1992; Deichaite, *et al.*, 1993; Berthiaume, *et al.*, 1994; Soltys, 1999; Corvi, *et al.*, 2001). Methylmalonate semialdehyde dehydrogenase is a mitochondrial enzyme that is acylated on its active site cysteine, presumably preventing this residue from interacting with substrate and therefore causing inhibition of the enzyme (Berthiaume, *et al.*, 1994). In mitochondria, fatty acylation may be nonenzymatic, since high levels of fatty acyl-CoA substrate can be generated during normal metabolic function and due to the absence of any ACBP in this organelle (Faergeman and Knudsen, 1997; Corvi, *et al.*, 2001).

1.2.3 Functions of palmitoylation

Palmitoylation promotes membrane anchoring, targeting of proteins to lipid rafts, trafficking proteins from the early secretory pathway to the plasma membrane and regulating protein activity.

Since palmitoylation occurs at membranes, soluble proteins must interact with membranes at least transiently to be palmitoylated. Once added, palmitate promotes stable membrane association.

Palmitoylation targets proteins to subdomains of the plasma membrane that are enriched in sphingolipids and cholesterol known as lipid rafts (Brown

and London, 1998; Edidin, 2003). Raft lipids are organized in the liquid-ordered phase in which the acyl chains of lipids are tightly packed, highly ordered, and extended (Schroeder, *et al.*, 1994; Brown and London, 1998). Proteins modified with saturated acyl chains have high affinity for the ordered lipid environment (Schroeder, *et al.*, 1994). Indeed, the content of lipid rafts is rich in proteins modified with fatty acyl chains. Proteins modified with glycosylphosphatidylinositol anchors are found on the cell surface, while proteins modified by tandem myristoylation and palmitoylation or tandem palmitoylation are localized to the cytoplasmic inner leaflet. Several studies show that the blockage of protein palmitoylation through site-directed mutagenesis (Shenoy-Scaria, *et al.*, 1994; Robbins, *et al.*, 1995; Arni, *et al.*, 1998; Guzzi, *et al.*, 2001) or by the use of the palmitoylation inhibitor 2-bromopalmitate (Webb, *et al.*, 2000), delocalizes proteins from rafts, highlight the importance that this modification has in targeting proteins to lipid rafts. In this regard, it has been suggested that signal transduction could be regulated by sequestering signaling proteins into different plasma membrane domains until they are brought together by an activating signal. In this manner, palmitoylation may regulate intracellular signaling pathways.

Palmitoylation is also involved in trafficking lipid-modified signal transducers. A classical example of the role of palmitoylation in trafficking signal transducers are the Ras proteins (Choy, *et al.*, 1999; Apolloni, *et al.*, 2000). All Ras proteins are synthesized on soluble ribosomes and farnesylated

in the cytoplasm by farnesyltransferase. Once in the endoplasmic reticulum (ER) Ras proteins encounter the next two processing enzymes, the *-CaaX* protease Ras-converting enzyme 1 (Schmidt, *et al.*, 1998) and the isoprenylcysteine carboxyl methyltransferase (Dai, *et al.*, 1998). It is at this point that Ras isoforms diverge in their trafficking pathways. While K-Ras4B, a nonpalmitoylated form of Ras, is directed to the plasma membrane, H-Ras and N-Ras are further modified by palmitoylation in the early secretory pathway. Blocking of H-Ras or N-Ras palmitoylation causes Ras to accumulate in the early secretory pathway, suggesting that palmitoylation is a signal to exit from the early secretory pathway. Newly synthesized heterotrimeric G proteins appear to use a pathway similar to that of palmitoylated Ras proteins to get to the plasma membrane (Michaelson, *et al.*, 2002; Takida and Wedegaertner, 2003).

Protein activity can also be modified by palmitoylation. Although the structural basis of this regulation is not well understood, *in vitro* assays are informative in the importance of palmitoylation in promoting or inhibiting protein interactions. G-protein-mediated signaling is regulated by palmitate at multiple levels. Palmitoylation regulates the localization of heterotrimeric G proteins at the plasma membrane and also regulates the transducing signal. Palmitoylation also impacts the interactions of G-protein α subunits and RGS proteins. RGS proteins constitute a large family with a shared RGS domain

that harbors GTPase activating protein (GAP) activity toward G_α subunits (Ross and Wilkie, 2000).

1.2.4 Acyl-Protein Thioesterase 1

In contrast to myristoylation, *in vivo* palmitoylation is a reversible modification. This fact stresses that a regulatory deacylation mechanism must exist. The dynamic of palmitoylation was suggested in pulse-chase experiments which showed the half-life of palmitate ranging from 20-180 min, far less than the half-life of the protein (Magee, *et al.*, 1987). The half-life of the palmitate moieties is significantly shorter than that of the protein, indicating that the complex goes through cycles of depalmitoylation and repalmitoylation. Removal of palmitate can occur either in response to signals or spontaneously (Robinson, *et al.*, 1995; Schweizer, *et al.*, 1996). Interestingly, for proteins involved in signal transduction, these cycles could be induced by activation of the protein, and thus, by controlling access to specific substrates. In this manner the signaling cascade could be regulated. Indeed an agonist-induced increase in palmitate turnover has been observed for the β2-adrenergic receptor, Gαs, endothelial nitric oxide synthase and H-Ras (Robinson, *et al.*, 1995; Huang, *et al.*, 1999; Baker, *et al.*, 2003). Palmitate turnover has also been described for the postsynaptic density protein PSD-95. It is known that the glutamate receptor activity triggers depalmitoylation of PSD-95 in neurons (El-Husseini Ael, *et al.*, 2002) by an unknown mechanism that seems to be dependent on the influx of extracellular calcium (El-Husseini Ael, *et al.*, 2002).

Palmitoyl-protein thioesterase I (PPT1) and PPT2 are lysosomal hydrolases involved in the cleavage of acyl-cysteine linkages during the process of protein degradation (Verkruyse and Hofmann, 1996; Soyombo and Hofmann, 1997). A second acyl protein thioesterase (APT1) that depalmitoylates G_α subunits and Ras has been cloned (Duncan and Gilman, 1998). APT1 is a 29 kDa cytosolic thioesterase that depalmitoylates proteins by catalyzing the cleavage of the thioester bond between the fatty acyl group and the protein. APT1 was originally purified as a lysophospholipase (Sugimoto, *et al.*, 1996), although the enzyme prefers palmitoylated protein substrates to lipid substrates (Duncan and Gilman, 1998). APT1 has a higher affinity and catalytic efficiency towards palmitoylated protein substrates and is widely conserved from yeast to humans (Duncan and Gilman, 1998). In addition, RGS4, H-Ras, and eNOS are substrates *in vitro* for mammalian APT1 (Duncan and Gilman, 1998; Yeh, *et al.*, 1999).

1.3 MITOCHONDRIA

Mitochondria are the cells' power sources. They are distinct organelles with two membranes: an outer and an inner mitochondrial membrane. These membranes define two submitochondrial compartments: the intermembrane space between the two membranes, and the matrix, or central compartment (Berg, *et al.*, 2002)(Figure 1.1). Fractionation and purification of these membranes and compartments has made it possible to determine their protein

and phospholipid compositions and to localize each enzyme-catalyzed reaction to a specific membrane or space.

The outer membrane defines the smooth outer perimeter of the mitochondrion which is quite permeable to most small molecules (with molecular masses of less than 5 kDa) and ions because it contains many copies of mitochondrial porin, a 30-35 kDa pore forming protein also known as VDAC, for voltage-dependent anion channel. VDAC plays a role in the regulated flux of metabolites, usually anionic species such as phosphate, chloride, organic anions, and the adenine nucleotides across the outer membrane. Some cytoplasmic kinases bind to VDAC, thereby obtaining preferential access to the exported ATP.

In contrast to the outer mitochondrial membrane, the inner membrane is freely permeable only to oxygen, carbon dioxide, and water. Protein constitutes 76 percent of the total inner membrane weight, a higher fraction than in any other cellular membrane. Cardiolipin (diphosphatidylglycerol), a lipid concentrated in the inner membrane, sufficiently reduces the membrane's permeability to protons, allowing a proton-motive force to be established between the intermembrane space and the matrix. Structurally, the inner mitochondrial membrane is very complex. Invaginations called cristae drastically increase the surface area and all of the complexes of the electron transport system, the ATP synthetase complex, and transport proteins are located in the membrane (Berg, *et al.*, 2002).

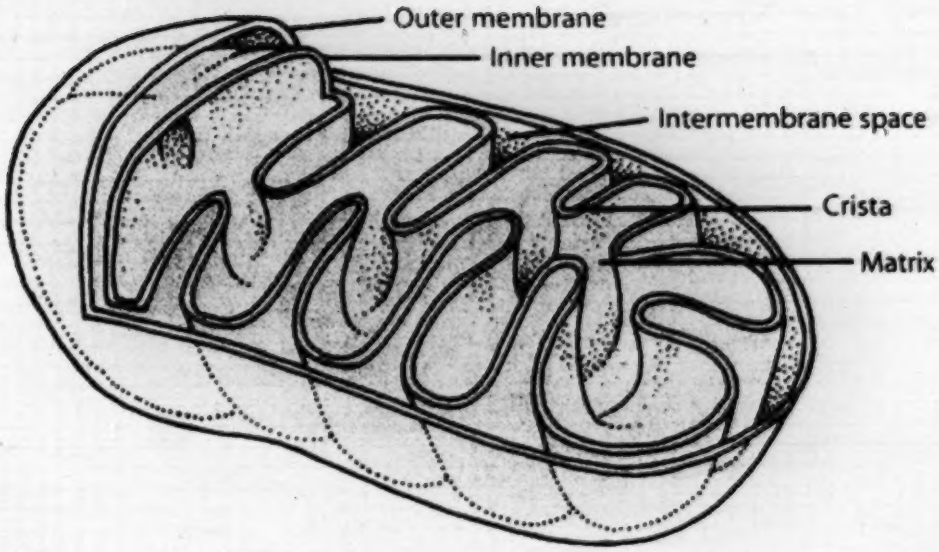


Figure 1.1 Diagram of mitochondria.

Diagram representing all the subcompartments of a mitochondrion. Scheme taken from http://www.cropsci.uiuc.edu/classes/cpsc112/Topicpages/respire.cfm.

As mentioned above, the membranes create two compartments. The intermembrane space, as implied, is the region between the inner and outer membranes. It has an important role in oxidative phosphorylation, the primary function of mitochondria. Contents of the matrix include the pyruvate dehydrogenase complex, most of the enzymes that catalyze fatty acid β-oxidation and the enzymes of the citric acid cycle (tricarboxylic acid cycle (TCA)) (the succinate dehydrogenase complex of the citric acid cycle is located in the inner membrane) generating reduced intermediates such as NADH that are reoxidized by the electron transport chain (ETC). The matrix also contains dissolved oxygen, water, carbon dioxide and the recyclable intermediates that serve as energy shuttles. Although diffusion is a very slow process, the folds of the cristae allow the matrix components to be in close contact with the inner membrane. Therefore, matrix components can diffuse to inner membrane complexes and transport proteins within a relatively short time (Berg, *et al.*, 2002). Two of the five enzymes involved in the urea cycle are also found in the mitochondrial matrix, with the remaining three residing in the cytosol.

1.4 UREA CYCLE

Urea is the principal nitrogenous waste of mammals. Most of our nitrogenous waste comes from the breakdown of amino acids, and this occurs by deamination. Deamination of amino acids results in the production of

ammonia (NH_3). Ammonia is an extremely toxic base, and its accumulation in the body would quickly be fatal. However, the liver contains a system of carrier molecules and enzymes called the urea cycle that quickly converts the ammonia (and carbon dioxide) into urea. Although our bodies cannot tolerate high concentrations of urea, it is much less poisonous than ammonia and is efficiently removed by the kidneys.

The stoichiometry of urea synthesis is:

$$CO_2 + NH_4^+ + 3\ ATP + aspartate + 2H_2O \rightarrow urea + 2\ ADP + 2\ P_i + AMP + PP_i + fumarate$$

The synthesis of fumarate by the urea cycle is important, because it links the urea cycle and the citric acid cycle (Figure 1.2). Fumarate is hydrated to malate, which is in turn oxidized to oxaloacetate. Oxaloacetate has several possible fates: (1) transamination to aspartate, (2) conversion into glucose by the gluconeogenic pathway, (3) condensation with acetyl-CoA to form citrate, or (4) conversion into pyruvate. A blockage of any of the four steps of the urea cycle has devastating consequences, because there is no alternative pathway for the synthesis of urea. The urea cycle in the liver plays an essential role in human as well as other ureotelic organisms to efficiently remove toxic ammonia from body tissues.

1.4.1 Urea cycle disorders

Urea cycle disorders include a group of diseases, each having a specific liver enzyme deficiency. These disorders vary in severity and may be first

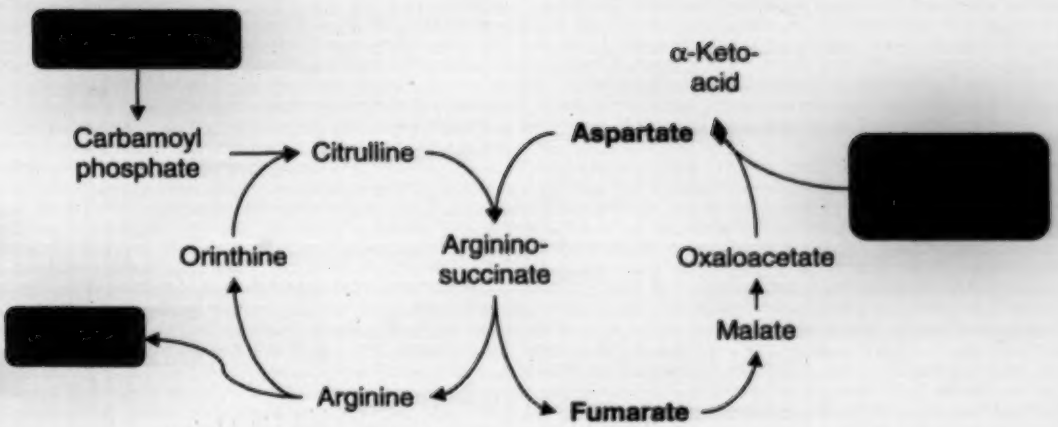


Figure 1.2. Metabolic integration of nitrogen metabolism.

The urea cycle, the citric acid cycle, and the transamination of oxaloacetate are linked by fumarate and aspartate. Adapted from Berg *et al.*, 2002.

detected at various ages, from newborn infants to adults. They lead to increased levels of ammonia in the blood, which may cause disturbed brain function and severe brain damage. Typical signs of the disease are decreased mental awareness, vomiting, combativeness, slurred speech, unstable gait and unconsciousness. The diagnosis of urea cycle disorders requires special laboratory tests. Because they are inherited, other family members may be affected.

1.5 CARBAMOYL PHOSPHATE SYNTHETASES

Carbamoyl phosphate synthetase (CPS) catalyzes the formation of the first intermediate in arginine and urea biosynthesis, carbamoyl phosphate. The CPS family can be grouped into three major classes according to their requirement for nitrogen donor and their allosteric activator N-acetyl-L-glutamate (AGA), which serves as a major regulatory feature of mammalian ammonia detoxification (Meijer, *et al.*, 1990). The CPS 1 group requires the allosteric activator AGA and utilizes ammonia as their nitrogen donor. Examples of this group are the rat and human liver CPSs. By contrast, members of the CPS 2 group do not require AGA and prefer glutamine as their nitrogen donor, but can also utilize ammonia. Examples of this group are the arginine-specific and pyrimidine-specific CPSs (CPS 2 located in the mammalian cytosol liver and the *E. coli* CPS of both pathways). CPS 3, like the CPS 1 group members requires AGA but, like CPS 2 prefers glutamine (it

can also use ammonia). CPS 3 is found in invertebrates (Tramell and Campbell, 1970; Tramell and Campbell, 1972), elasmobranch fishes (sharks and rays) (Anderson, 1991) and in some teleost fishes (Dkhar, *et al.*, 1991).

1.5.1 Carbamoyl Phosphate Synthetase 1

The CPS 1 reaction is the first and rate limiting step of the urea cycle (Krebs, *et al.*, 1973; Ratner, 1973). The reaction has been postulated to occur in three steps (Jones, 1965; Powers and Meister, 1978) and requires Mg^{2+}, K^+ and the allosteric activator AGA.

$$\text{Step 1:}\quad \text{MgATP} + HCO_3^- \xrightarrow[\text{AGA, CPS 1}]{Mg^{2+},\ K^+} \text{MgADP} + \text{carboxyphosphate}$$

$$\text{Step 2:}\quad \text{carboxyphosphate} + NH_3 \longrightarrow P_i + \text{carbamate}$$

$$\text{Step 3:}\quad \text{carbamate} + \text{MgATP} \longrightarrow \text{MgADP} + \text{carbamoylphosphate}$$

$$2\text{MgATP} + NH_3 + HCO_3^- \xrightarrow[\text{AGA, CPS 1}]{Mg^{2+},\ K^+} 2\text{MgADP} + P_i + \text{carbamoylphosphate}$$

CPS 1 consists of a single 165 kDa polypeptide chain (Clarke, 1976; Lusty, 1978) composed of a 40 kDa N-terminal domain and a 120 kDa C-terminal domain (Nyunoya, *et al.*, 1985). Even though originally discovered to be a liver mitochondrial matrix protein, there is a small fraction that has been shown to associate with the inner mitochondrial membrane (Powers-Lee, *et al.*, 1987). CPS 1 is also found in the mitochondria of the small intestine (Gamble and Lehninger, 1973; Clarke, 1976), although its levels are only one tenth those found in the liver (Ryall, *et al.*, 1985). The concentration of CPS 1 in the liver

mitochondrial matrix is estimated at 0.4 to 1.5 mM (Raijman and Jones, 1976; Meijer, *et al.*, 1990), representing 15-26 % of mitochondrial matrix protein and approximately 4 % of total liver protein (Clarke, 1976; Lusty, 1978; Raymond and Shore, 1981). Although it is an abundant protein, only one-third to one half of the enzyme is in its active form (Raijman and Jones, 1976).

CPS 1 is synthesized with a leader sequence that is cleaved upon entry of the 165 kDa precursor into the mitochondrion (Raymond and Shore, 1981) at residue 38 or 39 (Nyunoya, *et al.*, 1985). Consequently, the N-terminal boundary of the protein is residue 39 or 40.

CPS 1 requires AGA as an allosteric activator for its activity (Metzenberg, *et al.*, 1961; Fahien, *et al.*, 1964). It has been shown that one molecule of AGA binds one molecule of CPS 1 in a reversible and non-covalent manner (Alonso and Rubio, 1983). Although AGA does not participate directly in the CPS 1 reaction, it activates CPS 1 more than 50-fold by exposing an ATP-binding site (Rubio, *et al.*, 1983) and essential thiol (SH) groups within CPS 1 (Novoa, *et al.*, 1966; Marshall and Fahien, 1985). The AGA binding site is localized to the C-terminal region of CPS 1, as was shown by labeling experiments (Rodriguez-Aparicio, *et al.*, 1989; McCudden and Powers-Lee, 1996).

CPS 1 utilizes two molecules of ATP in its reaction: one molecule (ATP_B for Step 1) for the activation of bicarbonate and the other (ATPc for Step 2) for the phosphorylation of carbamate (Ratner, 1973; Rubio, *et al.*, 1983). These two sites appear as a consequence of an internal duplication of the gene

(Nyunoya, *et al.*, 1985; Powers-Lee and Corina, 1987). The presence of two distinctive sites was later confirmed by the use of the ATP analogues 8-azido-ATP and 5'-p-fluorosulfonylbenzoyladenosine (FSBA) (Powers-Lee and Corina, 1987; Potter and Powers-Lee, 1992). An increase in the affinity of the enzyme for ATP_B, was observed in the presence of AGA, but the binding affinity of ATPc to the CPS 1 was similar whether or not acetylglutamate was present (Rubio, *et al.*, 1983; Britton and Rubio, 1988).

CPS 1 contains a total of 21 cysteine residues of which none is involved in disulphide linkages (Clarke, 1976; Powers, 1981).

Regarding CPS 1 regulation, CPS 1 enzyme levels decline with protein-free diets and increase with starvation, high protein diets, administration of glucagon or glucocorticosteroids (Schimke, 1962; Schimke, 1962; Schimke, 1963; Snodgrass, *et al.*, 1978; Tsuda, *et al.*, 1979).

1.6 FATTY ACYLATION IN MITOCHONDRIA

Palmitoyl-CoA inhibits several mitochondrial enzymes including: rat adipocyte pyruvate dehydrogenase (PDH) (Moore, *et al.*, 1992), rat liver ADP/ATP translocase (Morel, *et al.*, 1974), bovine liver glutamate dehydrogenase (GDH) (Kawaguchi and Bloch, 1976), bovine liver methyl malonyl semialdehyde dehydrogenase (MMSDH) (Deichaite, *et al.*, 1993; Berthiaume, *et al.*, 1994) and rat liver CPS 1(Soltys, 1999).

1.6.1 Methylmalonyl Semialdehyde Dehydrogenase.

The mitochondrial protein methylmalonyl semialdehyde dehydrogenase (MMSDH), an enzyme of the valine and pyrimidine catabolic pathways, was shown to be fatty acylated on its active site cysteine residue (Cys319) thereby inhibiting its enzymatic activity (Deichaite, *et al.*, 1993; Berthiaume, *et al.*, 1994). It was demonstrated that MMSDH's fatty acylation varied with energy level, was reversible and specific for long chain fatty acyl-CoAs (Berthiaume, *et al.*, 1994). Fatty acylation of MMSDH required only the fatty acyl-CoA and seemed to be autocatalytic *in vitro* in apparently pure preparations of MMSDH. In support of the autocatalytic mechanism, recombinant MMSDH prepared from *E. coli* lysates was readily fatty acylated in the presence of [^{125}I]iodopalmitoyl-CoA (L.G.B. unpublished).

1.6.2 Glutamate Dehydrogenase

Bovine liver GDH is a 332 kDA mitochondrial matrix protein that catalyzes the reversible oxidative deamination of L-glutamate to α-ketoglutarate and ammonia (Golden and Frieden, 1971). Glutamate dehydrogenase is a branch point between carbon and nitrogen metabolism. It is an allosteric enzyme and is inhibited by both GTP and high concentrations of NADH, activated by ADP and AMP (Golden and Frieden, 1971). Palmitoyl-CoA was shown to dissociate the six-subunit active GDH to enzymatically inactive dimeric subunits and bind tightly to the dissociated enzyme (Kawaguchi and Bloch, 1976). Furthermore, palmitoyl-CoA was shown to bind to a cysteine residue in a covalent manner

(Berthiaume, *et al.*, 1994). In contrast to the mitochondrial enzyme MMSDH, bovine liver GDH does not utilize an active site cysteine residue to form a covalent intermediate during catalysis (Hudson and Daniel, 1993).

1.6.3 Carbamoyl Phosphate Synthetase 1

As mentioned earlier, CPS1 is the enzyme responsible for the first and rate limiting step of the urea cycle and thus, an important point of regulation. Furthermore, CPS 1 deficiency symptoms include coma, seizures, vomiting, severe hyperammonemia and death if treatment is not forthcoming or effective (Finckh, *et al.*, 1998). In cases of homozygous deficiency of CPS I, the ability to fix waste nitrogen is completely absent, which results in increasing levels of free ammonia with the attendant effects on the central nervous system (CNS)(Farriaux, *et al.*, 1977). CPS 1 deficiency highlights the importance of a proper regulation of this enzyme.

Soltys (1999) purified CPS 1 and showed it to be palmitoylated. Palmitate was shown to bind to CPS 1 on a cysteine residue/s to a cysteine residue in a covalent manner, inhibiting its acitivity. However, the mechanism by which palmitoylation inhibited CPS 1 was not clear (Soltys, 1999).

1.7. BIOORTHOGONAL COMPOUNDS AND STAUDINGER LIGATION

Molecular biological technologies and genetic methods are not directly applicable to the study of posttranslational modifications. Thus, there is an undeniable need to develop biochemical or chemical methods to identify

posttranslationally modified proteins and characterize the functions of such modifications. Proteomic analysis of posttranslationally modified proteins is usually challenging due to high complexity, and because there is no efficient method for their detection, quantification or proteomic analysis.

Bioorthogonal chemical reporters are non-native, non-perturbing chemical handles that can be modified in living systems through highly selective reactions with exogenously delivered probes. Proteins (Griffin, *et al.*, 1998; Zhang, *et al.*, 2003; Link, *et al.*, 2004; Chen, *et al.*, 2005), glycans (Mahal, *et al.*, 1997; Saxon, *et al.*, 2002; Luchansky, *et al.*, 2003; Luchansky, *et al.*, 2004) and lipids (Kho, *et al.*, 2004) have all been created with an assortment of chemical reporters in living cells and subsequently ligated with reactive probes. Chemical reactions that are selective and specific to a single chemical moiety in the context of complex biological milieu represent powerful tools for chemical analysis of biologically interesting molecules. An example of such a reaction is the Staudinger ligation pioneered by the Bertozzi group as a bioorthogonal reaction between a phosphine and an azide (Saxon and Bertozzi, 2000; Vocadlo, *et al.*, 2003). The reaction has unique features suitable for biological applications: first, the reaction can be carried out at room temperature in aqueous solution; second, there is no crossreaction with endogenous cellular compounds; third, neither phosphines nor azides occur in any known biomolecules; and finally, the phosphine can be engineered to incorporate a wide variety of tags including fluorescent probes and affinity tags

such as biotin, Myc and FLAG (Vocadlo, *et al.*, 2003; Kho, *et al.*, 2004; Prescher, *et al.*, 2004). Furthermore, the azide moiety is small, inducing minimum perturbation of substrate structure and is non toxic to the cell.

Although inorganic azide is toxic, organic azides are well tolerated during the time course required for labeling experiments; notably, azide-containing molecules have been used in therapeutics (e.g. 3'-azido-3'-deoxythymidine (AZT)) and several biological studies (Saxon, *et al.*, 2002; Chin, *et al.*, 2003).

1.7.1 Azido fatty acids for the detection and identification of palmitoylated proteins

The long exposure time required for monitoring the incorporation of [^{3}H]-fatty acids into proteins (1-3 months) has long impeded the progress of investigators working on protein fatty acylation.

At present there is not an efficient method for the selective identification and isolation of palmitoylated proteins that does not involve the use of radioactive compounds. Therefore, an efficient strategy for the enrichment of palmitoylated proteins will aid in the elucidation of the dynamic patterns and consequences of this modification. The method for the labeling of palmitoylated proteins would consist of three steps: metabolic labeling of proteins with an unnatural palmitate analogue in cells (azido-palmitate) or *in vitro* using its CoA derivative; chemoselective conjugation of azido palmitoylated proteins via the Staudinger ligation, which is highly specific between phosphine and azide, using a tagged phosphine capture reagent;

and detection and/or affinity purification of the resulting conjugated modified proteins. Global identification of the dynamically modified palmitoylated proteins would help determine how palmitoylation exerts its effects.

1.8. THESIS OBJECTIVES

As mentioned in section 1.6.3, CPS 1 activity is critical for the proper disposal of ammonia and its deregulation or total inactivity is incompatible with life. Palmitate was shown to bind to CPS 1 and, in this manner, to reduce its activity (Soltys, 1999). However, the mechanism by which palmitate exerts its effects on CPS 1 is not yet understood. Since one of the functions of palmitoylation is to regulate protein activity, this thesis explores the functional aspects of CPS 1 palmitoylation and expands our knowledge on the effect that this important posttranslational modification has on CPS 1 activity. In addition, we investigate if CPS 1 expression and/or activity are/is altered in physiological conditions such as obesity. Finally, we develop a technique to rapidly detect, isolate and identify other mitochondrial palmitoylated proteins using azido fatty acid analogues.

CHAPTER 2
MATERIALS AND METHODS

2.1 Materials

Reagents used in this thesis and their suppliers are summarized in table 2.1.

Acyl-CoA ligase, pyruvate kinase, lactate dehydrogenase, and Ultrogel AcA 34 were from Sigma-Aldrich (St. Louis, USA). Mono Q HR 5/5 column, [^{125}I]NaI (2.14 Ci/mmol) and ECL Plus detection system were from Amersham Biosciences (Uppsala, Sweden). Azidopalmitic acid was provided by Dr. Camille Falk (University of Texas Southwestern Medical Center, Dallas, TX, USA). The different tagged phosphine capture reagents were provided by Dr. Carolyn Bertozzi (University of California, Berkeley, California, U.S.A.). Mouse anti-Myc monoclonal antibody 9E10 was a gift from Dr. Robbins (University of Calgary, Alberta, Canada). PVDF membrane and Centricon filters were from Millipore Corp., U.S.A. The plasmids encoding for the chaperone system GroEL/GroES were kindly provided by Dr. Bernd Bukau (University of Heidelberg, Heidelberg, Germany)

Table 2.1 Reagents

Name	Source
5'-*p*-fluorosulfonylbenzoyladenosine (FSBA)	Sigma-Aldrich
Acrylamide	Bio-Rad
Adenosine 5'-triphosphate (ATP)	Sigma-Aldrich
Magnesium Chloride	Fisher
Ethylene Glycol-bis(beta-aminoethyl-ether)-N,N,N',N'-TetraAcetate (EGTA)	Fisher
Agar	Difco
Agarose, electrophoresis grade	Rose Scientific
Ammonium persulphate	Bio-Rad

Ammonium sulfate (enzyme grade)	Fisher
Ampicillin	Sigma-Aldrich
Bacto-tryptone	Difco
Bacto-yeast extract	Difco
Bis-acrylamide (N,N'- Methylene-bis-Acrylamide)	Bio-Rad
Bovine serum albumin (BSA) fraction V	Sigma-Aldrich
Bovine serum albumin (BSA) fatty acid free	Sigma-Aldrich
Bromophenol blue	BDH
Complete™ protease inhibitors	Roche Molecular Biochemicals
Coomassie Brilliant Blue R-250	ICN
Dimethyl sulphoxide (DMSO)	Sigma-Aldrich
Dimethylformamide (DMF)	BDH
Dithiothreitol (DTT)	Sigma-Aldrich
Ethanol	Commercial Alcohols
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich
Fatty acyl-CoAs	Sigma-Aldrich
Glacial acetic acid	Fisher
Glycerol	BDH
N-Acetyl-DL-glutamic acid (AGA)	Sigma-Aldrich
HEPES (N-Cyclohexyl-2-aminoethanesulfonic acid)	Invitrogen
Hydrochloric acid	Fisher
Isopropanol	Fisher
Isopropyl-β-D-thiogalactoside (IPTG)	Invitrogen
Kanamycin	Sigma-Aldrich
LB agar	Invitrogen
Leupeptin	Roche Molecular Biochemicals
Malt extract	Difco
Methanol	Fisher
N,N,N',N',-tetramethylenediamine (TEMED)	Invitrogen
N-ethylmaleimide	Sigma-Aldrich
Nonidet P-40 (NP40)/IGEPAL CA-630	Sigma-Aldrich
Percoll	Amersham Pharmacia Biotech
Phenylmethylsulfonyl fluoride (PMSF)	Sigma-Aldrich
Sodium chloride	Sigma-Aldrich
Sodium dodecyl sulphate (SDS)	Bio-Rad
Sodium hydroxide	Sigma-Aldrich
Sucrose	BDH
Tris base	Roche Molecular Biochemicals
Triton X-100	BDH

Tween 20 (polyoxyethylenesorbitan monolaureate)	Caledon
Yeast extract	Difco
Yeast nitrogen base without amino acids and ammonium sulfate	Difco

Table 2.2 Media and buffers

Name	Composition
5X SDS-PAGE loading buffer	300 mM Tris-HCl pH 6.8, 50% glycerol, 10% SDS, 0.1% Bromophenol Blue, 1 M DTT
K_2HPO_4 buffer	20 mM K_2HPO_4, pH 7.6, 1 mM DTT, 1 mM PMSF
IM buffer	250 mM mannitol, 5 mM HEPES pH 7.4, 0.5 mM EDTA, 0.1% albumin (essentially fatty acid free), 1 mM DTT, 1 mM PMSF, 20 µg/ml leupeptin
Hypotonic buffer	1 mM PMSF, 1 mM DTT, 20 µg/ml leupeptin
TD buffer	20 mM Tris-HCl, pH 8.0, 1 mM DTT
Lysis buffer	50 mM Tris-HCl pH 8.0, 1 mM EDTA, 100 mM NaCl, 1mM PMSF, 1 mg/ml lysozyme, 0.1 mg/ml DNAse, 0.5% TX-100
H buffer	300 mM saccharose, 5 mM HEPES, 200 µM EGTA, pH 7.2
PBS	1.4 M NaCl, 30 mM KCl, 10 mM Na_2HPO_4-$7H_2O$, 14 mM KH_2PO_4
YEPA	1% yeast extract, 2% peptone, 2% sodium acetate
LB	1% tryptone, 0.5% yeast extract, 1% NaCl
YNO	0.67% yeast nitrogen base without amino acids, 2 X CSM minus uracil, 0.05% Tween 40, 1% oleic acid
YNA	0.67% yeast nitrogen base without amino acids, 2% sodium acetate
TE buffer	10 mM Tris-HCl pH8.0, 1mM EDTA

Table 2.3 Primers used in PCRs

Name	Sequence
CPS 1-5'-60A2	5'-cccgggcatgcgaattcgtcgacccatatgacgaggattttgacagcttgcaaagtggtg-3'
CPS 1-3'-L	5'-caggggaccaaatgcacttcc-3'

CPS 1-5'-C	5'- gaacggtgtcaagatcatggg-3'
CPS-1-3'-K	5'-agaagccatctagatctcagaatagg-3'
CPS 1-5'-F	5'-cctattctgagatctgagatggcttct-3'
CPS 1-3'-I	5'-ggggtggcaacattgttg-3'
CPS 1-3'-J	5'-gccctcaccaaagctagccacctctcc-3'
CPS 1-5'-G	5'-ggagaggtggctagctttggtgagggc-3'
CPS 1-3'-E	5'-cctattctgagatctgagatggcttctactggagaggtggctagctttggtgagggc-3'
CPS 1-5'-mat	5'-ccccacgcgtcgacccatatgctttctgtgaaggcacag-3'

2.2 Animals

Male rats of the JCR:LA-cp strain, obese (cp/cp) and lean (cp/+) were raised in Dr. James C. Russell's established breeding colony at the University of Alberta (Russell, *et al.*, 1995). The animals were maintained in a controlled environment at 20°C and 50-55% relative humidity, with a 12:12 h light-dark cycle. Rat food (Teklad Rodent Diet; Harlan Sprague-Dawley Inc. Madison, WI) and distilled water were available *ad libitum*. This rat food is a corn- and wheat-based diet of 4% total lipid and 23% protein content, with energy content of approximately 3.3 kcal/g. For treatment purposes, drugs were incorporated into powdered diet that was then moistened, pelleted and air-dried. Animals were treated with either S15261 or Medica 16 as previously described (Russell, *et al.*, 2000; Atkinson, *et al.*, 2002). Liver tissue samples were removed and used immediately. All care of the animals and experimental procedures were in conformity with the guidelines of the Canadian Council on Animal Care and were subject to institutional review and approval.

2.3 Methods for Chapter 3

2.3.1 Preparation of radiolabeled palmitic acid

Radioiodination of the iodopalmitate with [^{125}I]NaI was performed as described in Berthiaume *et al.* (1995) without the high pressure liquid chromatography purification step. Typical specific activity of [^{125}I]iodopalmitate was 2 Ci/mmol. The final [^{125}I]iodopalmitate was aliquoted in glass vials each containing 500 µCi and dried.

2.3.2 Synthesis of the [^{125}I]iodopalmitoyl-CoA

Synthesis of the [^{125}I]iodopalmitoyl-CoA derivative was carried out using acyl-CoA ligase as reported previously (Berthiaume, *et al.*, 1995). Essentially, each [^{125}I]iodopalmitate-containing vial was resuspended in 5 µl of ethanol and incubated in presence of 5 mM ATP, 5 mM $MgCl_2$, 1 mM CoA, 0.3 units Acyl-CoA synthetase, 1 mM DTT, 0.1 mM EGTA and 0.5 % TX-100 (final concentrations) in 10 mM Tris-HCl pH 7.4 in a final volume of 500 µl for 4 h at 25°C.

2.3.3 *In vitro* fatty acylation reactions

Fatty acylation assays of CPS 1 in the presence of various substrates, cofactors, and reagents were carried out in a final volume of 50 µl using 2 µg of purified CPS 1 and 1 µCi of [^{125}I]iodopalmitoyl-CoA (10 µM final concentration) in 50 mM Tris-HCl, pH 7.6 (final concentration was achieved by

using a 250 mM Tris-HCl, pH 7.6, stock solution), for 30 min at 25°C. Typically, CPS 1 was preincubated with saturating concentration of reactants (2 mM AGA, 1.7 mM ATP) or 50 μM 5'-*p*-fluorosulfonylbenzoyladenosine (FSBA) containing 10 mM $MgCl_2$ or 50 μM palmitoyl- CoA or 1 mM *N*-ethylmaleimide (NEM) for 60 min at 25°C prior to addition of 1 μCi of [^{125}I]iodopalmitoyl-CoA (10 μM final concentration) for another 30 min at 25°C. Reactions were then stopped by the addition of 5X SDS-PAGE loading buffer (300 mM Tris-HCl, pH 6.8, 50% glycerol, 10% SDS, 0.1% Bromophenol Blue, 2 M DTT). Samples were incubated for 5 min at 95°C, loaded onto a 10% SDS-polyacrylamide gel (Laemmli, 1970), and electrophoresed at 15 mA. The gel was stained with Coomassie Blue, destained, dried for 2 h at 80°C using a gel dryer and exposed to autoradiographic film. The film was stored at -80°C, typically for 10 h. For reactions with FSBA or NEM, CPS 1 was previously dialyzed in 50 mM Tris-HCl, pH 7.6 for 16 h at 4 °C to remove DTT. FSBA was dissolved in dimethylformamide (Potter and Powers-Lee, 1992).

2.3.4 Purification of Carbamoyl Phosphate Synthetase 1

Rat liver CPS 1 was isolated at 4°C as described previously (Powers, 1981; Potter and Powers-Lee, 1992) with the following changes. Rat liver were rinsed with homogenization buffer H (20 mM Tris-HCl, pH 7.8, 10 % sucrose, 1 mM PMSF, 1 mM DTT), removed from the buffer and minced into small cubes. A ratio 3:1 (v:w) of isolation medium IM (250 mM mannitol, 5 mM HEPES, pH

7.4, 0.5 mM EDTA, 0.1 % fatty acid free albumin, 1 mM DTT, 1 mM PMSF, 20 µg/ml leupeptin) (Powers-Lee and Corina, 1986; Vance, 1990) to tissue was used and then homogenized using 5 strokes of the glass homogenizer with a Teflon pestle. The homogenate was centrifuged at 600 x g for 5 min at 4°C. The supernatant was filtered through a cheese cloth and then centrifuged at 10,500 x g for 30 min at 4°C in a JA 16 rotor. The final pellet enriched in mitochondria was suspended in IM buffer and stored frozen at -80°C in aliquots containing 1 g of total mitochondrial protein. For each preparation of CPS 1, 1 g of crude mitochondria aliquot was thawed and swollen in 90 ml of hypotonic buffer (1 mM PMSF, 1 mM DTT, 20 µg/ml leupeptin) for 30 min on ice. The suspension was then sonicated twice for 2 min at setting 2 on a Heat Systems sonicator (Farmingdale, NY USA). Nine ml of 200 mM Tris-HCl, pH 7.4 (10X), was then added to the solution, and the lysed mitochondria were centrifuged at 100,000 x g for 60 min in a Beckman Ti 45 rotor. The pellet was discarded. Solid ammonium sulfate was added to the supernatant to give a 35% saturated solution; the extract was stirred for 30 min at 4°C; the suspension was centrifuged at 20,500 x *g* for 20 min, and the pellet was discarded. Solid ammonium sulfate was added to the supernatant to make it an 80% saturated solution; the extract was stirred for 30 min at 4°C, and the suspension was centrifuged as above. The supernatant was discarded, and the pellet was resuspended in 3 ml of 0.3 M K_2HPO_4, pH 7.6, buffer and dialyzed for 2 h against K_2HPO_4 buffer (20 mM K_2HPO_4, pH 7.6, 1 mM DTT, 1

mM PMSF). The solution was loaded onto an Ultrogel AcA 34 packed in a XK 16/100 column equilibrated with 0.3 M K_2HPO_4, pH 7.6, 0.5 mM EDTA, 0.5 mM EGTA, 1 mM DTT, 1 mM PMSF, and 20 µg/ml leupeptin and run in an high performance liquid chromatography system (HPLC; Amersham Biosciences). The sample was eluted with the same buffer at 1 ml/min; 4-ml fractions were collected. Aliquots of chromatographic fractions were analyzed by 12% SDS-PAGE as described above.

The fractions from the gel filtration column containing CPS 1 were pooled and then equilibrated with TD buffer (20 mM Tris-HCl, pH 8.5, 1 mM DTT) by dialysis overnight at 4°C and applied to a Mono Q HR 5/5 column. The column was washed with 5 column volumes of TD buffer and eluted with a 2-ml gradient of 0–80 mM, followed by a 10-ml gradient of 80–300 mM, followed by a 2-ml gradient from 300 to 500 mM NaCl in TD buffer. One-ml fractions were collected at a flow rate of 0.5 ml/min. Aliquots of chromatographic fractions were analyzed by 12% SDS-PAGE as described above.

2.3.5 Enzymatic activity and protein determination

The enzymatic activity was measured by the pyruvate kinase/lactate dehydrogenase coupled assay (Raijman and Jones, 1976). Briefly, 20 µg of CPS 1 was incubated with the reaction mixture in a final volume of 1 ml at 25°C. This mixture contained 50 mM Tris-HCl, pH 7.6, 50 mM $KHCO_3^-$, 35 mM $(NH_4)_2SO_4$, 15 mM $MgSO_4$, 10 mM ATP, 5 mM phosphoenolpyruvate, 0.5 mM

NADH, 10 mM acetylglutamate, 35 units/ml pyruvate kinase, and 50 units/ml lactate dehydrogenase (final concentrations). The oxidation of NADH was determined by absorbance at 340 nm and then the activity was expressed as the amount of carbamoyl phosphate produced, assuming a stoichiometry of 1 mol of carbamoyl phosphate produced per 2 mol of ADP released (Figure 2.1) and a ε_{NADH}= 6.22 10^6 M^{-1} cm^{-1}. In order to study the time- and concentration-dependant CPS 1 inhibition, CPS1 was incubated at 25°C in the absence or presence of palmitoyl-CoA (5, 10, 20, 50, and 100 µM) or 50 µM FSBA with or without 2 mM AGA in 100 µl of 50 mM Tris-HCl, pH 7.6, buffer and assayed for enzymatic activity in a 1-ml final volume at different times (1, 5, 10, 30 and 60 min). For kinetic analysis, CPS 1 was incubated with 0, 20, or 50 µM of palmitoyl-CoA for 60 min in a 100-µl final volume and then assayed in the presence of different concentrations of AGA (0, 0.01, 0.05, 0.1, 0.5, and 1 mM), NH_4^+ (0, 0.2, 0.5, 1, 2, and 10 mM), HCO_3^- (0, 0.66, 1, 2.5, 5, 10, and 20 mM), and ATP (0, 1, 1.75, 2.5, 5, and 10 mM). While varying the concentrations of these substrates, the concentrations of other reagents in the CPS 1 assay were kept at the saturating concentrations indicated in the standard CPS 1 enzymatic assay mixture described above. In addition, CPS 1 was incubated with 0, 20, or 50 µM FSBA for 60 min in a final volume of 100 µl of 50 mM Tris-HCl pH 7.6 buffer prior to the spectrophotometric assay in the presence of different concentrations of ATP as described above.

$$2\,MgATP + NH_3 + HCO_3 \xrightarrow[AGA,\ CPS\ 1]{Mg^{2+},\ K^+} 2\,MgADP + P_i + carbamoylphosphate$$

$$PK:\ ADP + PEP \rightleftharpoons pyruvate + ATP$$

$$LD:\ pyruvate + NADH + H \rightleftharpoons lactate + NAD$$

Figure 2.1 Carbamoyl Phosphate Synthetase 1 activity determination.
The pyruvate kinase/ lactate dehydrogenase coupled assay was used for the determination of CPS 1 activity (Raijman and Jones, 1976)

Protein concentration was measured (Bradford, 1976) using 1 mg/ml bovine serum albumin as a standard.

2.3.6 CPS 1 substrate protection assay and fatty acyl-CoA specificity

CPS 1 was preincubated in the presence or absence of either 2 mM AGA (CPS 1 allosteric activator), 1.7 mM MgATP, or both for 60 min in 50 µl of 50 mM Tris-HCl, pH 7.6, and then either 50 µM palmitoyl-CoA or MgFSBA were subsequently added to the mixture and incubated for another 60 min in a total volume of 100 µl. The mixture was then assayed for enzymatic activity in a 1-ml reaction volume. In all assays, the concentration of $MgCl_2$ was kept constant at 10 mM. For specificity assays, CPS 1 was incubated with 50 µM coenzyme A, 50 µM acetyl-CoA, 50 µM palmitoyl-CoA, or 50 µM palmitate for 60 min and then assayed for enzymatic activity. All incubations were carried out at room temperature (25°C). Protein concentration was measured (Bradford, 1976) using bovine serum albumin as a standard.

2.3.7 Reversibility assay for the binding of palmitoyl-CoA to CPS 1

Purified CPS 1 (2 µg) was preincubated with 1 µCi (10 µM) of [^{125}I]iodopalmitoyl-CoA for 2 h at 25 °C in 40 µl of 50 mM Tris-HCl, pH 7.6. After this period, CPS 1 was further incubated in the absence or presence of non-labeled palmitoyl-CoA (10, 20, 50, 100, and 200 µM) dissolved in 50 mM Tris-HCl, pH 7.6, for 1 h at 25°C in a total volume of 50 µl. Reactions were

stopped by the addition of 5X SDS-PAGE loading buffer and processed as for SDS-PAGE and autoradiography as described above.

2.3.8 Obtention of an anti-CPS 1 serum

Antibodies against CPS 1 were raised in rabbits as described (Harlow and Lane, 1988). Briefly, a rabbit was injected 4 times at 15-day intervals with a mixture of 375 µg of purified CPS 1 (1mg/ml) with incomplete Freund's adjuvant in a final volume of 1 ml (1:1; v:v) half intramuscularly and half subcutaneously. Blood samples (1ml) were taken 10 days post-injection and antibody presence was performed by using the sera obtained in western blots, using purified CPS 1 as protein sample. The specificity of the antiserum was established by performing western blots with lysed mitochondria. Terminal bleeding was performed by cardiac puncture. Serum was obtained by blood incubation for 2 h at 4°C and centrifugation for 10 min at 3,500 rpm. The supernatant (serum) was aliquoted in 1ml-eppendorff tubes and stored at -80°C.

2.3.9 Isolation of intact mitochondria from livers of JCR:LA-cp rats

Livers were removed rapidly, rinsed with cold H buffer (300 mM saccharose, 5 mM HEPES, 200 µM EGTA, pH 7.2), and cut into small pieces. The tissues were resuspended in H buffer in a ratio 5:1 (v:w) and homogenized in a Potter-Thomas homogenizer with 20 strokes at about 500

rpm. The homogenates were centrifuged for 10 min at 800 x g. The supernatants were recovered and the pellets were washed in 10 ml of H buffer and centrifuged as described above. The two supernatants were combined and centrifuged for 10 min at 8750 x g. The pellets were loaded on top of a discontinuous Percoll gradient 60%/30%/18% and centrifuged for 10 min at 8750 x g. Purified mitochondria are recovered from the lower interface (between 60% and 30% Percoll), diluted 10X in H buffer and centrifuged for 10 min at 6800 x g to remove the Percoll, which is toxic for mitochondria. The supernatants are discarded and the mitochondria are resuspended in 1 ml IM buffer (see table 2.2 for buffer composition).

Aliquots of the purified mitochondria were diluted 1:100, solubilized in 1% TX-100 and assayed for protein concentration as mentioned before.

2.3.10 CPS 1 immunoblot

Purified mitochondrial proteins (1 µg) were separated on a 10% SDS-PAGE, run at 15 mA and transferred onto a polyvinylidene fluoride (PVDF; Millipore) membrane for 2 h at 0.7 A. Membranes were blocked for 2 h in 5% non-fat dried milk, 0.1% (v/v) Tween 20 in PBS (see table 2.2 for buffer composition). Then the membrane was probed for CPS 1 using our rabbit anti-CPS 1 polyclonal antibody at a 1:10,000 dilution (dissolved in the same blocking solution) for 1 h, washed twice with PBS (see table 2.2 for buffer composition), twice with PBS-Tween 20 and again with PBS. Then the

membrane was incubated with a HRP-conjugated sheep anti-rabbit IgG (1:5,000 dilution). All the previous steps were performed at 25°C with constant agitation. Finally the membranes were processed for ECL Plus detection (Amersham Bioscinces).

2.3.11 Primary culture of hepatocytes

Primary cultures of hepatocytes were obtained from Dr. Richard Lehner laboratory. The primary culture of hepatocytes were obtained by the method of collagenase perfusion technique, as described (Yao and Vance, 1988). Briefly, the rats were anesthetized by intraperitoneal injection of pentobarbitone (5 mg/100 g body weight). The liver was infused through the portal vein first with 40-50 ml Ca^{2+}- and Mg^{2+}-free Hanks' solution (Gibco) supplemented with 20 mM glucose, 25 mM Hepes, 0.5 mM EGTA, 10 µg/ml insulin, then with 50-60 ml of collagenase solution (6 mg/ml enzyme dissolved in Hank's solution containing Ca^{2+} and Mg^{2+}) at 5 ml/min. After perfusion the liver was excised, chopped with scissors, and gently shaken with 10 ml of collagenase solution at 37°C to break cell clumps. The cell suspension was filtered through a metal mesh and 40 ml DMEM (Life Technologies, Grand Island, NY) was added to stop the enzyme reaction. The cells were washed three times with 40 ml of medium and plated on culture dishes (5 x10^6 cells/dish) in 10 ml of medium.

2.3.12 Metabolic labeling of primary culture of hepatocytes with [^{125}I]iodopalmitate

Metabolic labeling of primary culture of hepatocytes was performed as described (Zhao, *et al.*, 2000). Briefly, primary cultures of hepatocytes were starved for 1 h by incubation with 10ml of 10 µg/ml of BSA fatty acid free in DMEM (Life Technologies, Grand Island, NY). After this period, the cells were incubated for 4 h with 3 ml of 100 µCi of [^{125}I]iodopalmitate previously conjugated to 30 µg BSA. Then, the cells were subjected to cell fractionation as described (Berthiaume, *et al.*, 1994). Aliquots of all fractions were loaded onto a 10% SDS-polyacrylamide gel (Laemmli, 1970), and electrophoresed at 15 mA. The gel was stained with Coomassie Blue, destained, dried for 2 h at 80°C using a gel dryer machine and exposed to autoradiographic film. The film was stored at -80°C, typically for 10 h.

2.4 Methods for Chapter 4

2.4.1 Cloning of CPS 1 in pCMV5

CPS 1 cloning into pCMV5 was performed following standard molecular biology protocols (Sambrook, *et al.*, 1989). Briefly, the cDNA of CPS 1 was obtained from ATCC (plasmid pHN 3491; ATCC# 63195) and PCR was performed using the oligonucleotides CPS 1-5'-60A2 (see table 2.3 for sequence) and CPS 1-3'-L (see table 2.3 for sequence) (Figure 2.2). The amplified fragment was digested with the generated endonuclease restriction

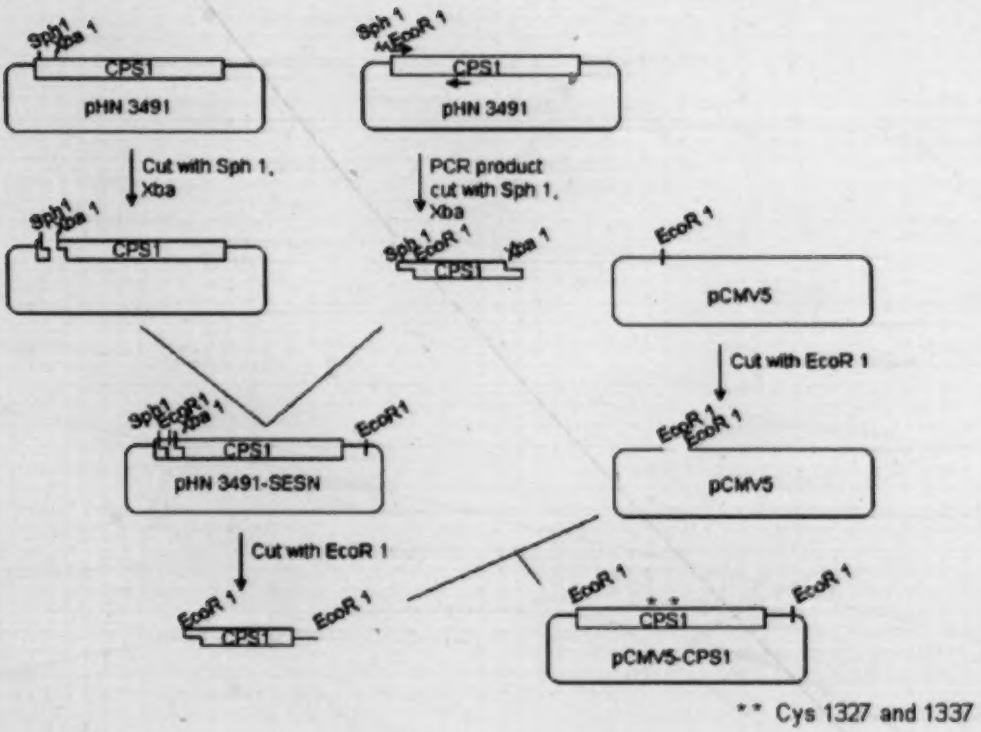


Figure 2.2 Generation of pCMV5-CPS 1 construct.

CPS 1 cDNA was amplified by PCR and the amplified fragment was digested with the generated endonuclease restriction sites SphI (on the 5') and XbaI (on the 3'). In parallel, pHN 3491 was linearized by cutting with SphI and XbaI and the fragment obtained by PCR was ligated into the linearized vector, generating the plasmid pHN3491-SESN. The new generated plasmid was cut with EcoRI and CPS 1 was isolated; pCMV5 was also cut with EcoRI and the fragment containing CPS 1 was ligated into it.

sites SphI (on the 5') and XbaI (on the 3'). In parallel, pHN 3491 was linearized by cutting with SphI and XbaI and the fragment obtained by PCR was ligated into the linearized vector, generating the plasmid pHN3491-SESN. The generated plasmid was cut with EcoRI and CPS 1 was isolated; pCMV5 was also cut with EcoRI and the fragment containing CPS 1 was ligated into it.

CPS 1 mutant constructs C1327S, C1337S and C1327/1337S were generated by splicing by overlap extension strategy (Horton, *et al.*, 1989). This method joins two DNA fragments by first amplifying them by means of polymerase chain reactions (PCR) carried out on each template DNA using oligonucleotide primers designed so that the ends of the resultant PCR products contain complementary sequences (Figure 2.3). When the two PCR products are mixed, denatured and reannealed, the single-stranded DNA strands having the complementary sequences anneal and then act as primers for each other. Extension of the annealed area by DNA polymerase produces a double-stranded DNA molecule in which the original molecules are spliced together. Gene splicing by overlap extension (SOE), provides for recombining DNA molecules at precise junctions irrespective of nucleotide sequences at the recombination site and without the use of restriction endonucleases or ligase. The SOE approach is a fast and simple way of recombining and modifying nucleotide sequences. Table 2.3 shows the primer pairs used to generate the different constructs. In order to generate the C1327S mutant the pair of primers [CPS 1-5'-C and CPS 1-3'-K] and [CPS 1-5'-F and CPS 1-3'-I] were

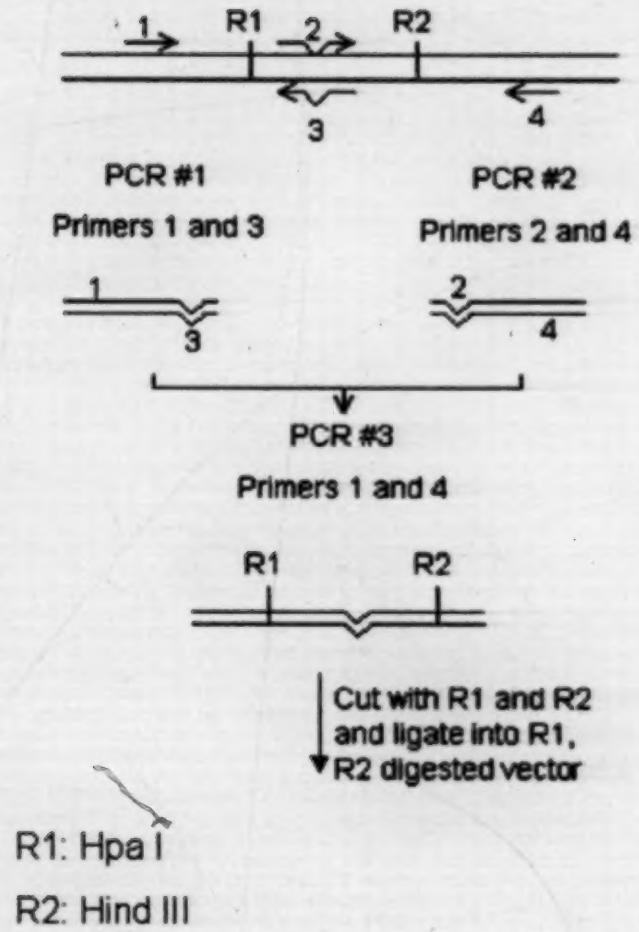


Figure 2.3 Splicing by overlap extension.

Diagram showing the main characteristics of the technique as described by Horton *et al.* (1989).

used for the first PCR and finally, the pair of primers [CPS 1-5'-C and CPS 1-3'-I] were used for the second PCR. The C1337S mutant was generated by using the pair of primers [CPS 1-5'-C and CPS 1-3'-J] and [CPS 1-5'-G and CPS 1-3'-I] in the first PCR and finally, the pair of primers [CPS 1-5'-C and CPS 1-3'-I] were used for the second PCR. In order to generate the C1327/1337S mutant the pair of primers [CPS 1-5'-C and CPS 1-3'-K] and [CPS 1-5'-E and CPS 1-3'-I] were used for the first PCR and finally, the pair of primers [CPS 1-5'-C and CPS 1-3'-I] were used for the second PCR. For all constructs, the final PCR product was digested with HpaI (site upstream of the generated mutation) and Hind III (downstream to the generated mutation) endonucleases. The wild type pCMV5-CPS 1 was also digested with HpaI and Hind III and the fragment containing the mutations was ligated and thus, the mutant constructs were generated.

2.4.2 Generation of stable cell lines in McArdle RH-7777

Rat hepatoma McArdle RH-7777 cells were seeded at $6x10^6$ cells per 100 mm-diameter dish. The cells were grown in Dulbecco's modified Eagles Medium (DMEM; Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) and penicillin-streptomycin (100 IU/ml-100 µg/ml, Invitrogen). The cells were transfected with 4 µg of the appropriate CPS 1 constructs (cloned in pCMV5) and 0.3 µg of pSV (neomycin resistance; ratio 20:1 mol/mol) using Fugene 6 (Roche Molecular Biochemicals) as a

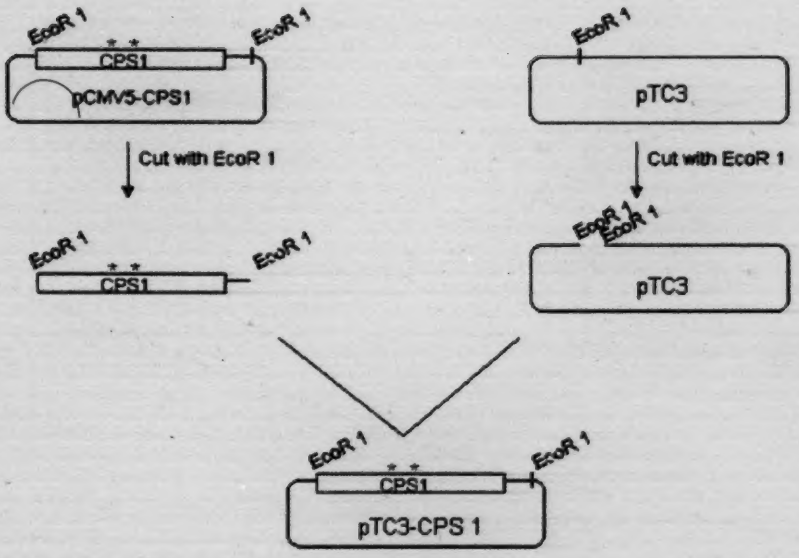
transfection reagent. Stable cells were cultivated in DMEM supplemented with 400 µg/ml G418 (Invitrogen) passing them every 2 days using 0.25% trypsin/ 1ml EDTA wash. After 1 month, stable cells were tested for CPS 1 expression by western blot. All cells were cultivated at 37°C in a humidified 5% CO_2 incubator.

2.4.3 Transient transfection in COS-7 cells

COS-7 cells were seeded at $6x10^6$ cells per 100 mm-diameter dish. The cells were grown in Dulbecco's modifed Eagles Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and penicillin-streptomycin (10,000 IU/ml-10,000 mg/ml, Invitrogen). The cells were transfected with 4 µg of the appropriate CPS 1 constructs (cloned in pCMV5) using Fugene 6 (Roche Molecular Biochemicals) as a transfection reagent. Cells were passed twice using 0.25% trypsin/ 1ml EDTA wash before being tested for CPS 1 expression by western blot. All cells were cultivated at 37°C in a humidified 5% CO_2 incubator. COS-7 cells below passage 16 were utilized in all transfections.

2.4.4 Subcloning of CPS 1 constructs in pTC3

The pCMV5-CPS 1 construct was digested with EcoRI and the CPS1 coding insert was purified (Figure 2.4). pTC3 was also linearized with EcoRI and the CPS 1 insert was ligated into it, transformed into chemically



** Cys 1327 and 1337

Figure 2.4 Generation of pTC3-CPS 1 construct.

The pCMV5-CPS 1 construct was digested with EcoRI and CPS1 coding insert was purified. pTC3 was also linearized with EcoRI and the CPS 1 insert was ligated into it.

competent DH5α and grown on LB media supplemented with 100 μg/ml of ampicillin. Ampicillin resistant clones were purified by miniprep protocol, using a miniprep kit (Promega, Madison, USA) and analyzed for the presence of CPS 1 cDNA insert in the proper orientation. The final vector was named pTC3-CPS 1. pTC3 encodes for uracil (ura) auxotrophy marker.

2.4.5 Transformation of *Yarrowia lipolytica*

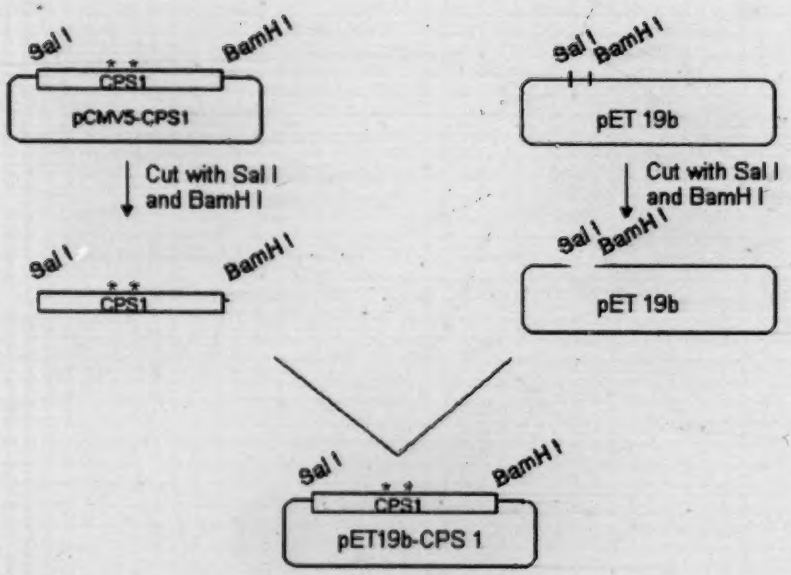
The strain of *Y. lipolytica* used was E122: genotype MATA, ura 3-302, leu 2-270, lys 8-11.

Transformation of *Y. lipolytica* was essentially as described by Nuttley et al. (Nuttley, *et al.*, 1993). Basically, YEPA (1% yeast extract, 2% peptone, 2% sodium acetate) was inoculated with *Y. lipolytica* cells and grown for approximately 16 h in a rotating wheel at 30°C. The entire cultures were subcultured into YEPA and grown to an optical density of approximately 1 at λ=600 nm. Cells were collected by centrifugation, resuspended in TE buffer pH 7.5 (see Table 2.2 for buffer composition) and 1 M lithium acetate, and incubated for 30 min at 30°C with agitation (250 rpm), at which time 1 M DTT was added. Incubation was continued at 100 rpm for 15 min. Cells were collected, washed once with room-temperature water, once with ice-cold water, and once with ice-cold 1 M sorbitol, and resuspended in a minimum of 1 M sorbitol. The purified DNA (see section 2.4.4) was added and the cells were subjected to electroporation with a Cell-Porator (Gibco) at 250 V and 16 kΩ

and rescued into ice-cold sorbitol. The cells were then spread onto YNA plates (0.67% yeast nitrogen base without amino acids, 2% sodium acetate) without uracil as a selection media and incubated at 30°C, in order to select the colonies that incorporated the plasmid.

2.4.6 CPS 1 expression in *Yarrowia lipolytica*

In a first attempt to test if *Y. lipolytica* expressed CPS 1, the cells were grown in YNA media without uracil (see table 2.2 for composition) until optical density=0.8 at λ=600 nm. Then the cells were subcultured in YNO supplemented with oleic acid for different amount of times. The cells were centrifuged at 3000 rpm for 10 min and washed once with room-temperature water. The cells were pelleted down by centrifugation and resuspended in LB (see table 2.2 for buffer composition) and 425-600 mm glass beads were added and the cells were disrupted using a Beadbeater 8 device (BioSpec Products, Bartlesville, USA). Cells were subjected to three cycles of bead beating on the maximum setting (30 seconds beating followed by 1 min on ice per cycle). Lysates were subsequently cleared by centrifugation (1,000 x g) for 15 min at 4°C and the supernatants were loaded onto a 10% SDS-PAGE and western blot analysis was performed as described above.



** Cys 1327 and 1337

Figure 2.5 Generation of pET19b-CPS 1 construct.

pCMV5-CPS 1 was digested with SalI and BamHI and the CPS1 insert was purified (Figure 2.5). The expression vector pET 19b was also digested with SalI and BamHI and the CPS 1 insert was ligated to it to generate pET19b-CPS 1.

2.4.7 Subcloning CPS 1 constructs in bacterial expression vectors

pCMV5-CPS 1 was digested with SalI and BamHI and the CPS1 insert was purified (Figure 2.5). The expression vector pET19b was also digested with SalI and BamHI and the CPS 1 insert was ligated to it to generate pET19b-CPS 1. The ligated DNA (pET19b-CPS 1) was amplified in DH5α cells and purified using a miniprep kit (Promega, Madison, USA). In order to express CPS 1, AD494 (DE3) cells were electroporated with pET19b-CPS 1 and plated on LB agar supplemented with 100 µg/ml ampicillin. AD494 (DE3) cells provide a thioredoxin reductase mutation, which allows disulfide bond formation to occur, providing the potential to produce properly folded, active proteins (Derman, *et al.*, 1993). CPS 1 is a nuclear encoded protein that is targeted to the mitochondria through a mitochondrial target import sequence. In order to test whether the passage through a membrane was important for CPS 1 proper folding, a mature version of CPS 1 was constructed and expressed in the pET12c vector. The pET 12c has an OmpT leader sequence that directs the protein to the periplasmic space. This passage through a membrane may be important and may resemble the passage to get CPS 1 into the mitochondria. In order to obtain the mature version of CPS 1, a PCR using the primers CPS 1-5'-mat and CPS 1-3'-L (see table 2.3 for sequence). The 5' primer has an endogenous SalI cutting site and the amplified PCR product also contains a BamHI site at the 3' end (Figure 2.6). The PCR

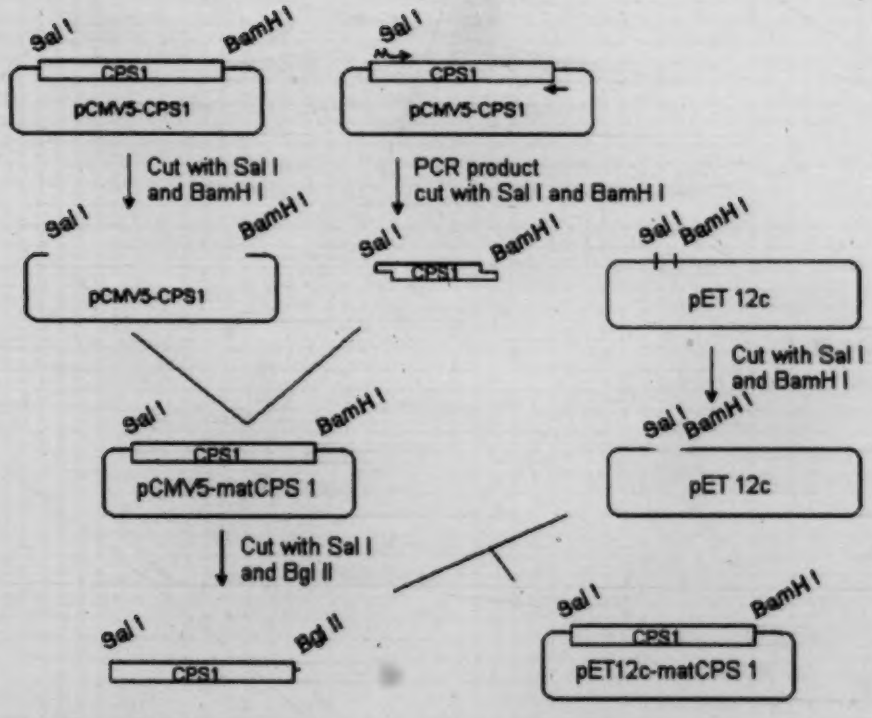


Figure 2.6 Generation of pET12c-CPS 1 construct.

The mature version of CPS 1 was obtained by PCR using the primers CPS 1-5'-mat and CPS 1-3'-L (see table 2.3 for sequence). The 5' primer has an endogenous Sal I cutting site and the amplified PCR product also contains a BamH I site at the 3' end. The PCR product was cut with Sal I and BamH I and purified. pCMV5-CPS 1 was also digested with Sal I and BamH I and the CPS1 insert from the PCR was ligated into it to generate the plasmid pCMV5-matCPS 1. This plasmid (pCMV5-matCPS 1) was digested with Sal I and BglI I and the matCPS 1 insert was purified. The expression vector pET12c was digested with Sal I and BamH I, and the CPS 1 insert was ligated to it.

product was cut with SalI and BamHI and purified. pCMV5-CPS 1 was also digested with SalI and BamHI and the CPS1 insert from the PCR was ligated into it to generate the plasmid pCMV5-matCPS 1. This plasmid (pCMV5-matCPS 1) was digested with SalI and BglII and the matCPS 1 insert was purified. The expression vector pET12c was digested with SalI and BamHI, and the CPS 1 insert was ligated to it. The DNA was amplified by transforming chemically competent DH5α with each construct and grown on LB supplemented with 100 μg/ml ampicillin. The DNA of positive clones containing pET12c-matCPS 1 was purified. BL21 (DE3) cells were transformed with pET12c-matCPS 1 and plated in LB agar plates supplemented with 100 μg/ml of ampicillin.

2.4.8 CPS 1 expression in bacteria

Positive clones were cultured in LB media containing 100 μg/ml ampicillin until optical density=0.5 at λ= 600 nm and induced with 1 mM IPTG for 4 h. The cells were pelleted, resuspended in lysis buffer (see table 2.2 for composition), centrifuged at 10,000 x g for 10 min and the supernatant and pellet were obtained. The pellet was resuspended in the same volume of lysis buffer and aliquots of both fractions were loaded onto a 10% SDS-PAGE to test CPS 1 expression. The pellet contains inclusion bodies.

The same experiments were performed with both full length CPS 1 and the mature version of it, but co-expressing the chaperone system GroEL/GroES.

In order to do this, the plasmids pBB528 (containing the cDNA for lacIq and resistance to chloramphenicol) and pBB541 (containing the cDNA for GroESL and resistance to spectinomycin) were transferred to BL 21(DE3) cells (mature CPS 1) or to AD494 (DE3) cells (full length CPS 1). Analysis of CPS 1 expression and solubility was performed as described above.

2.4.9 Mass spectrometry analysis

CPS 1 (20 μg) was incubated in presence or absence of 50 μM palmitoyl-CoA in a final volume of 50 μl for 30 min at 25°C. The samples were immediately taken to the Alberta Cancer Board Proteomics Mass Spectrometry Facility (Department of Chemistry, University of Alberta) to perform mass spectrometry analysis. The samples were digested with trypsin followed by acetonitrile/water extraction. No reduction or alkylation steps were performed. The peptides were analyzed by MALDI-MS and MS/MS. In order to preserve the lipid containing peptides, all processes were carried out under extreme caution using glass vials, pipettes and syringes. Peptide extracts were analyzed on a Bruker REFLEX III (Bremen/Leipzig, Germany) TOF mass spectrometer using MALDI in positive ion mode. Obtained peptide masses were compared with CPS 1 theoretical digests for a possible palmitoylation site. A shift in 238 m/z was expected for a palmitoylated peptide and the MS/MS spectra were manually interpreted.

2.4.10 Partial protease digestions

CPS 1 (6 μg) was incubated with [^{125}I]iodopalmitoyl-CoA for 30 min at 25°C in 20 μl of 50 mM Tris-HCl, pH 7.6. After this period, CPS 1 was further incubated with either 300 ng elastase or 1200 ng trypsin for another 30 min at 25°C in 40 μl of 50 mM Tris-HCl, pH 7.6. The reactions were stopped by addition of 2 μl of 10 mM elastatinal (for elastase) or 1 μl of 20 mg/ml leupeptin (for trypsin) and samples were kept on ice for 5 min. For serial digestions, the first partial digestion was stopped with the specific protease inhibitor and then the second partial digestion took place under the same conditions. Samples were then subjected to SDS-PAGE, stained, destained, dried and exposed onto a film or phosphorimager cassette.

2.5 Methods for Chapter 5

2.5.1 Preparation of lysed mitochondria

Mitochondrial fractions were prepared from Sprague-Dawley rats (300- 350 g) liver by differential centrifugation as described in section 2.3.2. The final pellet enriched in mitochondria (1 gram) was swollen in 90 ml of hypotonic buffer (1 mM PMSF, 1 mM DTT, 20 μg/ml leupeptin) for 30 min on ice. The suspension was then sonicated twice for 2 min at setting 2 on a Heat Systems sonicator. Nine ml of 1 M HEPES, pH 7.4, was then added to the solution, and the lysed mitochondria were centrifuged at 100,000 x g for 60 min in a

Beckman Ti45 rotor. The pellet was discarded and the supernatant was stored in 500 µl-aliquots with protease inhibitors and 20 % glycerol.

2.5.2 CPS 1 purification

CPS 1 was purified to apparent homogeneity as described in section 2.3.2. Briefly, lysed mitochondria were obtained as described above. Lysed mitochondria were subjected to 40-80 % ammonium sulphate precipitation, gel filtration and anion exchange columns. The final purified CPS 1 sample was 1.16 mg/ml with a specific activity of 0.30 µmol of carbamoyl phosphate produced per min per mg of CPS 1 at 25°C, which is in agreement to that previously reported (Corvi, *et al.*, 2001).

2.5.3 Preparation of radiolabeled and azidopalmitoyl-CoA

Radioiodination of iodopalmitate with [^{125}I]NaI and synthesis of [^{125}I]iodo palmitoyl-CoA derivatives were performed as described in sections 2.3.1 and 2.3.2 respectively. The synthesis of azidopalmitoyl-CoA was carried out exactly as the [^{125}I]palmitoyl-CoA analogue (section 2.3.2), but substituting the [^{125}I]palmitic acid by azidopalmitic acid (azidoC_{14} or N_3C_{14}).

2.5.4 Labeling of proteins with [^{125}I]iodopalmitoyl-CoA

Incubations were carried out in a final volume of 50 µl using 10 µg of lysed mitochondria with 50 µM final concentration of [^{125}I]iodopalmitoyl-CoA as

described by Corvi *et al.* (2001). The reaction was then stopped by the addition of 5X SDS-PAGE loading buffer. Samples were incubated for 2 min at 100°C and loaded onto a 10% SDS-polyacrylamide gel. Radiolabel incorporation into proteins was visualized by autoradiography.

2.5.5 Labeling of proteins with azidopalmitoyl-CoA

Incubations were carried out in a final volume of 50 µl using either 10 µg of lysed mitochondria or 1 µg of purified CPS 1 with 60 µM final concentration of azidopalmitoyl-CoA in 100 mM HEPES-NaOH, pH 8.0, for 2 h at 25 °C in the dark. After this incubation period, the Myc-tagged phosphine capture reagent was added in a 2:1 mol:mol ratio to that of azidopalmitoyl-CoA and the reaction was allowed to proceeded for another 4 h. The reaction was then stopped by the addition of 5X SDS-PAGE loading buffer.

2.5.6 Detection of azidopalmitoylated proteins

Samples were incubated for 2 min at 100°C, loaded onto a 10% SDS-PAGE, electrophoresed and finally the proteins were transferred to a PVDF membrane for 2 h at 0.7 A. Membranes were probed for the incorporation of the fatty acid with a mouse anti-Myc monoclonal antibody 9E10 at a 1:2,000 dilution and a HRP-conjugated sheep anti-mouse IgG (1:5,000 dilution). Detection of the flag-phosphine capture reagent was performed by using an anti-flag monoclonal antibody at a 1:6,000 dilution and a HRP-conjugated

sheep anti-mouse IgG (1:5,000 dilution). Finally the membranes were processed for ECL Plus detection and then were stained with Coomassie Blue R-250. The fluorescein-phosphine capture reagent was detection was carried out by scanning the SDS-PAGE gel on a STORM 840 from Molecular Dynamics (Sunnyvale, CA, USA).

2.5.7 Neutral hydroxylamine treatments

CPS 1 (1 µg) or lysed mitochondria (10 µg) were labeled with the azidopalmitoyl-CoA and the Myc-phosphine capture reagent as described above. Then, either 1M Tris-HCl, pH 7.0, 1M NH_2OH-NaOH, pH 7.0, or 0.2 M NaOH (final concentrations) was added to the samples and the reactions were allowed to proceed at room temperature in the dark. Samples treated with neutral Tris-HCl and NH_2OH were incubated for 16 h. The samples treated with NaOH were allowed to proceed for 1 h (Bizzozero, 1995). The NaOH reaction was stopped by neutralization with HCl followed by addition of 5X SDS-PAGE loading buffer. The reactions with neutral NH_2OH and Tris-HCl were stopped by addition of 5X SDS-PAGE loading buffer and the azidofatty acylated proteins were detected as described above.

2.5.8 N-ethylmaleimide treatment

DTT was removed from the buffer containing CPS 1 by ultracentrifugation by using Centricon filters and washed 3 times with 500 µl of 100 mM HEPES,

pH 7.4. Either 10 µg of lysed mitochondria or 1 µg of purified CPS 1 were incubated in presence or absence of 10 mM N-ethylmaleimide for 1 h at room temperature in a final volume of 50 µl. Then the azidopalmitoyl-CoA and the Myc-tagged phosphine capture reagent were added as described above. The reaction was stopped and the detection of the azidofatty acylated proteins was carried out as described above.

2.5.9 MS analysis of azidofatty acylated proteins

100 µg of lysed mitochondrial proteins were labeled with or without azidopalmitoyl-CoA, followed by incubation in presence or absence of Myc-phosphine capture reagent as described above and the samples were split in two sets and loaded on a 10% SDS gel for either western blot detection (10 µg) or Coomassie Blue staining (90 µg). Azidopalmitate incorporation was visualized by western blot as described above and bands that were azidopalmitoylated and sufficiently separated from the each other were cut out from the gel and sent for MS analysis.

2.5.10 Hydroxylamine and N-ethylmaleimide treatments data quantitation

Data was quantified by ImageQuant Software (Molecular Dynamics, Sunnyvale, California, USA). The results were expressed as percentages respect to control treated lane.

CHAPTER 3

Regulation of Carbamoyl Phosphate Synthetase 1 *in vitro* and *in vivo*

A version of this chapter has been published. Corvi M.M., Soltys C.-L. M. and Berthiaume L.G. (2001). Regulation of mitochondrial Carbamoyl-phosphate Synthetase 1 activity by active site fatty acylation. *J. Biol. Chem.* **276**, 45704-45712.

3.1 Overview

As was mentioned in Chapter 1, dynamic protein fatty acylation has been postulated to play a potential role in the regulation of amino acid catabolism (Deichaite, *et al.*, 1993; Berthiaume, *et al.*, 1994). In mitochondria, enzymes that are part of different catabolic pathways must compete for a common pool of reduced coenzyme A, NAD^+, and FAD^+ cofactors and for a common electron transport chain (Brass and Beyerinck, 1988). As such, the variable availability of such cofactors, appropriate catabolites, and the activity of the competing enzymes can affect the rate of a given catabolic pathway.

The mitochondrial protein methylmalonyl semialdehyde dehydrogenase (MMSDH) was shown to be fatty-acylated on its active site cysteine residue thereby inhibiting its enzymatic activity (Deichaite, *et al.*, 1993; Berthiaume, *et al.*, 1994). Because the extent of fatty acylation of MMSDH varied with mitochondrial energy level, was reversible, and was specific for long-chain fatty acyl-CoAs, it was proposed that fatty acylation of MMSDH could act as a novel mode of regulation of enzymatic activity. In addition, as a way to prioritize substrate degradation, this novel mode of regulation has been proposed to mediate a metabolic cross-talk between amino acid and fatty acid catabolism (Deichaite, *et al.*, 1993; Berthiaume, *et al.*, 1994). Thus, protein fatty acylation of a few select mitochondrial proteins could perhaps be regulated by intramitochondrial levels of long-chain fatty acyl-CoAs.

Upon incubation of mitochondria with radiolabeled fatty acids, several proteins have been shown to incorporate fatty acids in a covalent manner in mitochondria isolated from rat liver and COS-7 cells (Stucki, *et al.*, 1989; Berthiaume, *et al.*, 1994), but to date, only two fatty-acylated mitochondrial proteins have been identified, MMSDH and CPS 1 (Berthiaume, *et al.*, 1994; Soltys, 1999). However, the mechanism by which palmitate acts on CPS 1 remains unclear.

The purpose of this chapter is to characterize CPS 1 inhibition by palmitoyl-CoA. This will be carried out by in vitro experiments using purified CPS 1 as well as by *in vivo* studies using obese and lean rats.

3.2 Inhibition of CPS 1 Catalytic Activity by Palmitoyl-CoA and FSBA

In order to investigate the effect of palmitoyl-CoA on CPS 1, CPS 1 enzymatic activity was measured in the presence of increasing concentrations of palmitoyl-CoA for variable periods of time (Figure. 3.1). We found a time- and concentration-dependent decrease of CPS 1 activity and showed that concentrations of palmitoyl-CoA well within physiological mitochondrial concentrations (up to 230 μM) (Faergeman and Knudsen, 1997) can inhibit CPS 1 catalytic activity. In more detail, incubation of CPS 1 with 100 μM palmitoyl-CoA for 60 min inhibited 89 ± 2% (n = 4) of CPS 1 catalytic activity, whereas incubation of CPS 1 with as little as 5 μM palmitoyl-CoA resulted in a 22 ± 3% (n = 4) reduction in activity. In comparison, preincubation of CPS 1

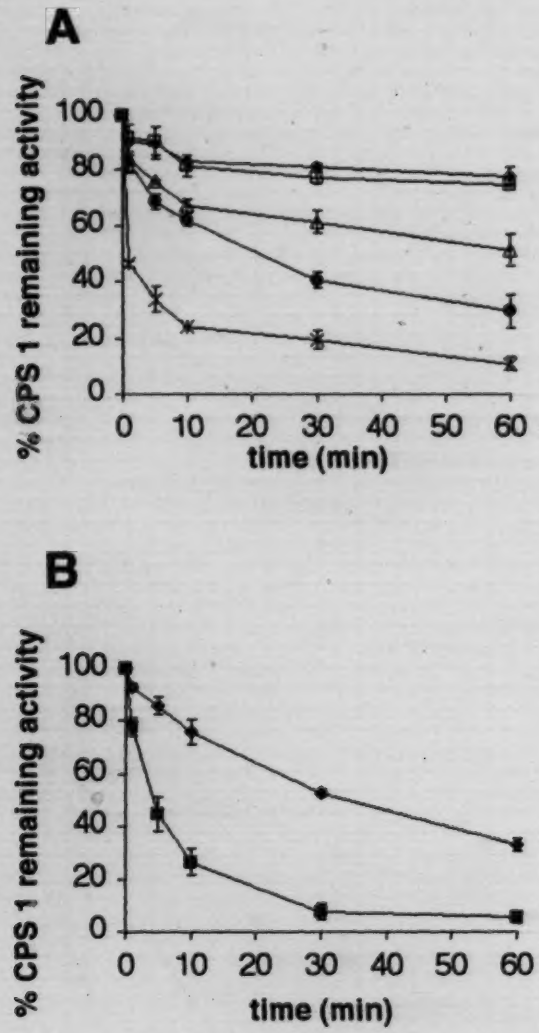


Figure 3.1 Effect of incubation of CPS 1 in the presence of palmitoyl-CoA or FSBA on CPS 1 enzymatic activity.

CPS 1 (20 μg) was preincubated in the presence of 5 (♦), 10 (□), 20 (Δ), 50 (●), and 100 (X) μM palmitoyl-CoA in the absence of AGA (*A*) or 50 μM FSBA in either the presence (■) or absence (□) of AGA (*B*) for variable times and then assayed for CPS 1 activity as described under "Materials and Methods."

with 50 μM FSBA, a known ATP analog that selectively reacts with CPS 1 active site cysteine residues (Marshall and Fahien, 1985) in the presence or absence of the allosteric activator AGA, resulted in inactivation of 94 ± 2 (n = 4) and 67 ± 2% (n = 4) of CPS 1 activity, respectively.

3.3 CPS 1 Kinetic Analysis

Since CPS 1 activity was significantly reduced by incubation with palmitoyl-CoA, we decided to perform some kinetic studies to investigate the type of inhibition that palmitoyl-CoA exerts on CPS 1. The reduction in CPS 1 activity was reflected in kinetic assays at a variable concentration of the substrates and AGA by a decrease in *Vm* without substantial effect on *Km* values (Figure 3.2). The concentration of palmitoyl-CoA that, when incubated with the enzyme for 60 min, decreases the V*m* to one-half is defined here as *Ki* value and was found to be 18, 15, and 24 μM when the varied substrates were MgATP, NH_4^+, or HCO_3^-, respectively. *Km* for MgATP extrapolated from Fig. 3.2, *A* and *E*, was 1.0 mM in both cases, which is in accordance with that of Lusty (Lusty, 1978). *Km* for AGA was 0.38 mM (Fig. 3.2*B*), for NH_4^+ was 0.21 mM (Fig. 3.2*C*), and for HCO_3^- was 1.9 mM (Fig. 3.2*D*), and values were within the range reported by other groups (Raijman and Jones, 1976; Lusty, 1978; Powers, 1981). The rate of CPS 1 activity reduction by 50 μM palmitoyl-CoA in the absence of AGA was faster than that observed with 50 μM under the same conditions, suggesting that palmitoyl-CoA is a better inhibitor than

FSBA. This is confirmed by our calculated inhibitory constants (*Ki*) as the *Ki* determined for FSBA was 131 ± 20 μM (in the absence of AGA) and for palmitoyl-CoA the *Ki* was 19 ± 5 μM.

Based on our kinetic results, palmitoyl-CoA inhibition of CPS 1 corresponded to an irreversible inactivation of the enzyme.

3.4 Specificity of CPS 1 inhibition

In order to test if the inhibitory effect on CPS 1 was specific or not, CPS 1 was incubated with either 50 μM coenzyme A, 50 μM acetyl-CoA, or 50 μM palmitate. All this compounds did not modify CPS 1 activity, whereas under the same conditions, 50 μM palmitoyl-CoA reduced CPS 1 activity 63 %, suggesting that the inhibitory effect of palmitoyl-CoA requires a long-chain fatty acid activated in its CoA derivative and does not simply reflect the high reactivity of some cysteine residue(s) (Fig. 3.3*A*).

3.5 Protection of CPS 1 activity reduction by Palmitoyl-CoA or FSBA by various substrates and cofactors

In order to test if the acylation site on CPS 1 was localized in the active site, we decided to perform substrate protection assays. If palmitoylation takes place in the active site, preincubation of CPS 1 with substrates can reduce or prevent acylation. As can be seen in Figure 3.3B, the remaining activity after we preincubated CPS 1 with palmitoyl-CoA in the absence of other ligands or

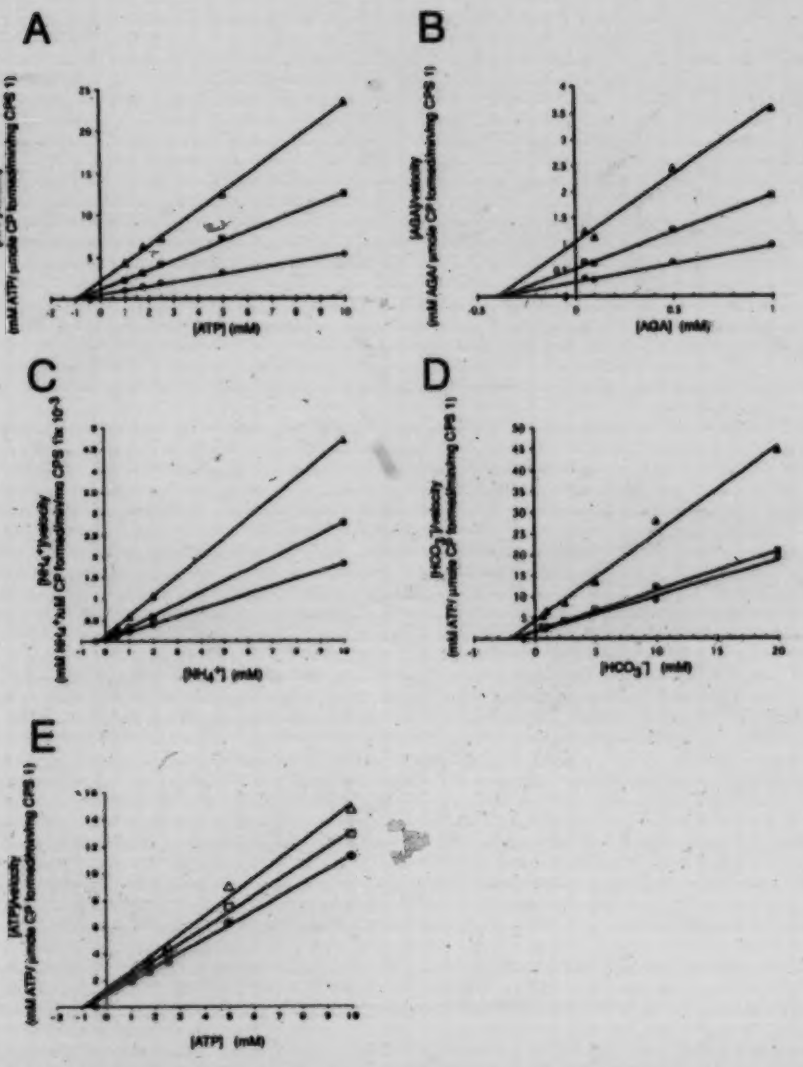


Figure 3.2 Hanes-Woolf plot of CPS 1 inhibition by palmitoyl-CoA and FSBA in the presence of various substrates.

Apparent irreversible inactivation of CPS 1 activity by palmitoyl-CoA with different concentrations of ATP (*A*), AGA (*B*), NH_4^+(*C*), and HCO_3^- (*D*) or by FSBA in presence of ATP (*E*). Kinetic studies were performed as described under "Materials and Methods" using 20 µg of CPS 1 per assay. Control (•), 20 (■) and 50 µM (▲) palmitoyl-CoA and 20 (□), and 50 µM (Δ) FSBA.

in the presence of AGA was ~30% ((31 ± 6% (+ AGA) *vs* 31 ± 3% (- AGA) of control; $n = 6$), whereas the remaining activity found in the presence of AGA and MgATP was 76 ± 6% ($n = 6$). The remaining activity after we preincubated CPS 1 with FSBA in the presence AGA and MgATP was 36 ± 2% ($n = 4$) of the controls, respectively. Whereas AGA by itself did not protect against inactivation of CPS 1 by palmitoyl-CoA, AGA stimulated the FSBA inactivation rate of CPS 1 (3.3 ± 3% (+ AGA) *vs* 21 ± 4% (- AGA) of control; $n = 4$) (Fig. 3.3*B*). These studies show that CPS 1 fatty acylation may be occurring at the active site. Furthermore, since FSBA is known to react with active site cysteine residues 1327 and 1337 and since FSBA preincubation drastically decreases CPS 1 palmitoylation levels, we feel confident that fatty acylation of CPS 1 is likely occurring on at least one of the CPS 1 active site cysteine residues.

3.6 Fatty Acylation of CPS 1 in the Presence of Substrates, Allosteric Activator, and FSBA

CPS 1 catalyzes the three-step conversion of HCO_3^-, NH_4^+, and ATP into carbamoyl phosphate in the presence of the allosteric activator *N*-acetylglutamate (AGA). To test whether formation of complexes between the enzyme and ATP, or its allosteric activator AGA, would interfere with the fatty acylation process of CPS 1, we preincubated CPS 1 with saturating concentrations of reactants (2 mM AGA, 1.7 mM MgATP) prior to addition of

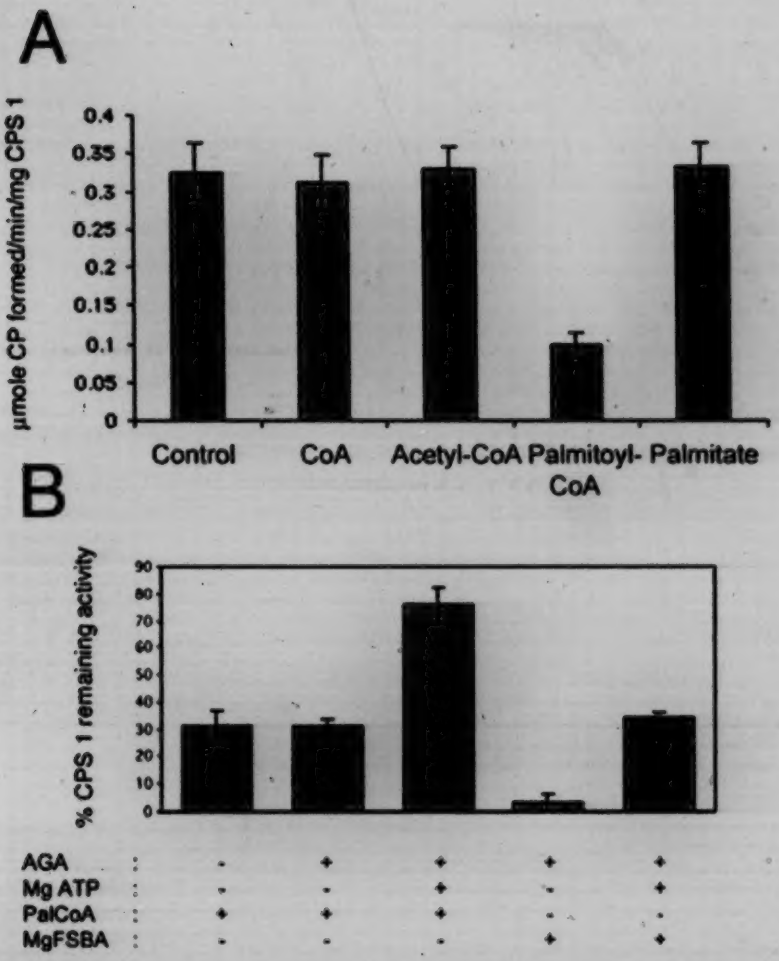


Figure 3.3 Palmitoyl-CoA specificity and substrate protection assays.

CPS 1 (20 µg) was incubated with 50 µM coenzyme A, 50 µM acetyl-CoA, 50 µM palmitoyl-CoA, or 50 µM palmitate for 60 min and then assayed for enzymatic activity as described under "Materials and Methods" (*A*). In substrate protection assays, CPS 1 was preincubated for 60 min in the presence of AGA (2 mM) or MgATP (1.7 mM) and then incubated for 60 min in the absence or presence of 50 µM palmitoyl-CoA (PalCoA) or 50 µM MgFSBA and then assayed for enzymatic activity as described under "Materials and Methods" (*B*). 100% activity was considered as the activity remaining in an identical incubation in which palmitoyl-CoA or FSBA was absent.

[^{125}I]iodopalmitoyl-CoA (Fig. 3. 4). When CPS 1 was first incubated individually with reactants and then radiolabeled with the [^{125}I]iodopalmitoyl-CoA, AGA and ATP treatment reduced CPS 1 acylation by 55 ± 2 and 39 ± 2% (n = 4), thus leaving 45 ± 2 and 61 ± 2% non-acylated enzyme, respectively. Addition of AGA and ATP together leads to a near complete prevention of fatty acylation of CPS 1 (86 ± 4% reduction). To test the possibility that CPS 1 may be acylated on its active site cysteine residue(s), we preincubated CPS 1 with FSBA in the presence or absence of AGA. Preincubation of CPS 1 with FSBA and AGA, which leads to a selective inactivation of two essential cysteine residues (Marshall and Fahien, 1985), almost completely prevented the acylation of CPS 1 (greater than 95% inhibition). This exciting result suggested that fatty acylation of CPS 1 was very likely occurring at the active site of the enzyme. In the absence of AGA, the extent of FSBA protection against acylation was not as strong (60% inhibition of acylation), suggesting once again that binding of AGA facilitated the binding of the ATP analog FSBA to CPS 1 active site and thus increased the protection of some active site-related cysteine residues against acylation observed when both are present. However, palmitoylation can occur on a distal cysteine residue that can alter the three-dimensional structure of CPS 1 and thus lead to an inhibition of its activity. Preincubation with 50 μM palmitoyl-CoA for 60 min, prior to the addition of radiolabeled [^{125}I]iodopalmitoyl-CoA, resulted in a 63 ± 2% (n = 4)

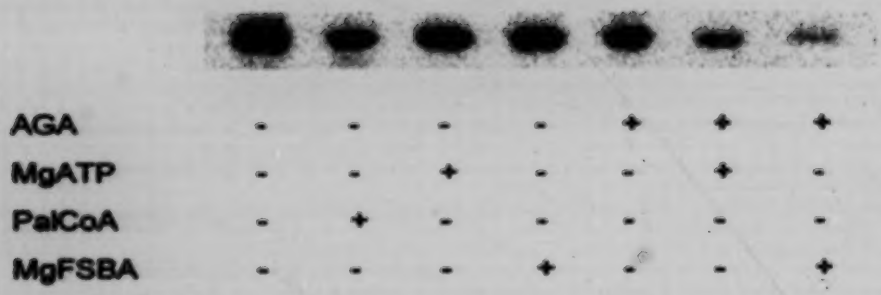


Figure 3.4 CPS 1 fatty acylation protection assays.

CPS 1 (2 μg) was preincubated for 60 min in the presence, as indicated, of 2 mM AGA, 1.7 mM MgATP, 50 μM palmitoyl-CoA (PalCoA), or 50 μM MgFSBA and then incubated for 60 min with [^{125}I]iodopalmitoyl-CoA as described under "Materials and Methods." Autoradiogram of the 12% SDS-PAGE is shown.

reduction in labeling in comparison to the control. Interestingly, this result correlates with the kinetic results obtained in Fig. 3.1, in which we showed that a 60-min preincubation of CPS 1 with 50 μM palmitoyl-CoA resulted in 37% remaining CPS 1 activity. These results link CPS 1 inactivation with fatty acylation.

3.7 Reversibility of CPS 1 acylation

To test if palmitoyl-CoA binds to CPS 1 in a reversible manner, we labeled CPS 1 to saturation with [^{125}I]iodopalmitoyl-CoA and then incubated it for 1 h in the presence of different concentrations of palmitoyl-CoA. As shown in Fig. 3.5, the label on CPS 1 could not be displaced even in the presence of 200 μM palmitoyl-CoA. This result indicates that the attached palmitate binds in an apparent irreversible manner under these conditions of excess of fatty acyl-CoAs *in vitro*.

3.8 Generation of a CPS 1 antiserum

For many experiments we needed to detect CPS1 in a complex mixture of proteins. The only antibody available was raised in Dr. Powers-Lee laboratory, but it cross-reacted with many other proteins. So, we decided to produce our own CPS 1 antisera in rabbits. Our rabbit antiserum was more specific for CPS 1 detection by western blot than that of Dr. Powers-Lee at the same dilutions when it is exposed to purified CPS 1 (Figure 3.6). Our CPS1

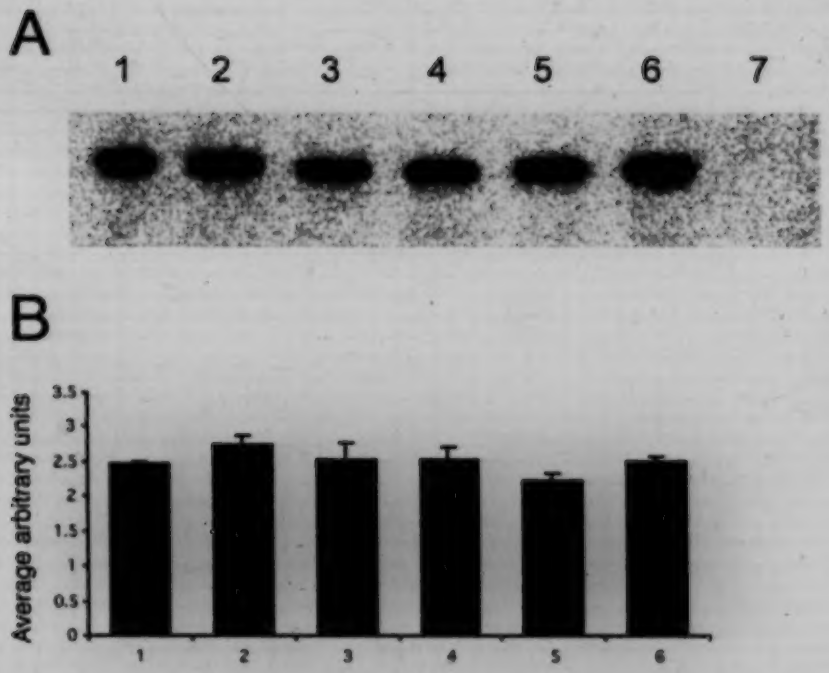


Figure 3.5 Reversibility of CPS 1 fatty acylation.

CPS 1 (2 μg) was labeled with 8 μM [^{125}I]iodopalmitoyl-CoA for 2 h and then further incubated with additional cold palmitoyl-CoA at different concentrations for 1 h. Autoradiogram *(A)*. Quantitation of *A* in arbitrary phosphorImager units ± S.E. of 2 independent experiments *(B)*. *Lane 1*, no palmitoyl-CoA; *lane 2*, 10 μM palmitoyl-CoA; *lane 3*, 20 μM palmitoyl-CoA; *lane 4*, 50 μM palmitoyl-CoA; *lane 5*, 100 μM palmitoyl-CoA; *lane 6*, 200 μM palmitoyl-CoA. *A*, *lane 7* is a control for nonspecific binding of [^{125}I]iodopalmitoyl-CoA to CPS 1 in which CPS 1 was incubated for 1 s in the presence of [^{125}I]iodopalmitoyl-CoA, and the reaction was immediately stopped by addition of sample buffer.

antiserum is also very efficient in the detection of CPS 1 in complex protein mixtures, in immunoprecipitation assays and in immunofluorescence studies (data not shown).

3.9 Protein fatty acylation in primary cultures of rat hepatocytes

In general, mitochondrial fatty acylation is tested by incubation of purified mitochondria with a mitochondrial acylation buffer and radiolabeled palmitic acid, [^{125}I]iodopalmitate (Soltys, 1999; Corvi, *et al.*, 2001). The results of these experiments are generally questioned as they are performed *in vitro*. In order to test palmitoylation *in vivo*, we labeled primary culture of hepatocytes with [^{125}I]iodopalmitate and then performed cellular fractionation. Figure 3.7 shows that the palmitate is incorporated onto proteins not only in the cytosol, but also in mitochondria (Figure 3.7, P10 fraction). Importantly, CPS 1 appeared to incorporate the radiolabeled fatty acid. The profile of *in vivo* palmitoylated proteins acylated in mitochondria is very similar to the one obtained in the *in vitro* assay using purified mitochondria (Corvi, *et al.*, 2001), making the *in vitro* system a valid method to study palmitoylation of mitochondrial proteins.

3.10 CPS 1 expression and activity under different physiological conditions

With the purpose of investigating if our *in vitro* studies correlated with *in*

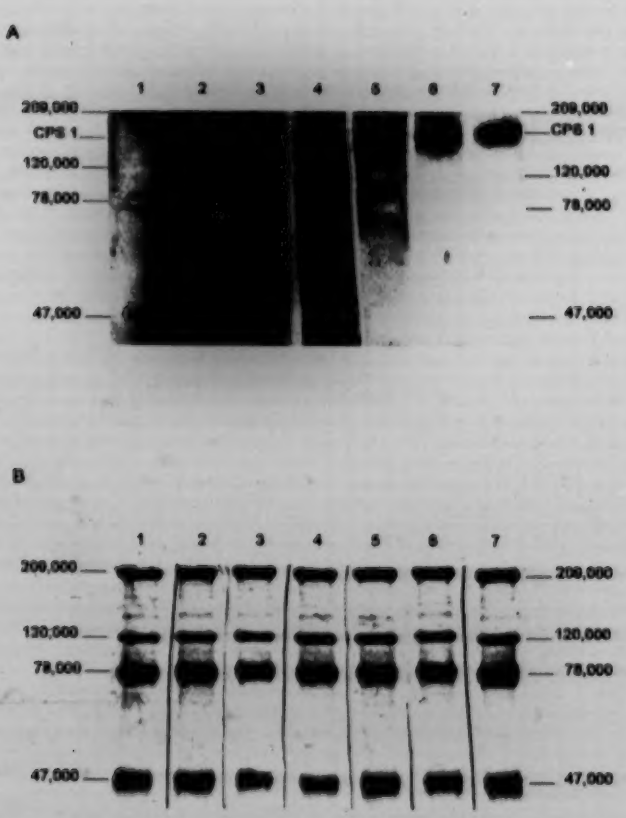


Figure 3.6. Development of an anti-CPS 1 antiserum.

Western blot for the detection of purified CPS 1. Lane1: pre-immune serum (1:500); Lane 2: Powers-Lee antibody anti-CPS 1 (1:500); Lane 3: Powers-Lee antibody anti-CPS 1 (1: 1,000); Lane 4: Powers-Lee antibody anti-CPS 1 (1: 2,000); Lane 5: Our antibody anti-CPS 1 (1:500); Lane 6: Our antibody anti-CPS 1 (1: 1,000); Lane 7: Our antibody anti-CPS 1 (1: 2,000). Panel B: Coomassie blue staining of PDVF membrane (A). All the lanes were loaded with 100 ng of purified CPS 1 and 10 µl of molecular weight standards (B; New England Biolabs, Ipswich, USA).

vivo data, we decided to study CPS 1 expression and activity in liver mitochondria of lean and obese rats.

JCR:LA-cp rats are a model of obesity/insulin resistance syndrome (Russell, *et al.*, 1989). The cp gene has recently been identified as a mutation leading to a stop codon in the extracellular domain of the leptin receptor (Wu-Peng, *et al.*, 1997). This leads to the absence of any leptin receptor in the plasma membrane of cp/cp rats. JCR:LA-cp rats are insulin resistant and obese, although plasma glucose levels are not significantly different than the lean counterpart (Atkinson, *et al.*, 2002). Free fatty acid levels and triacylglycerols (both in plasma and in liver) are also elevated in JCR:LA-cp rats (Elam, *et al.*, 2001; Atkinson, *et al.*, 2002). This increase is due to an elevated endogenous fatty acids synthesis (Elam, *et al.*, 2001). Acetyl-CoA carboxylase activity and expression as well as fatty acid synthase mRNA are increased in liver and thought to be responsible for the high content of fatty acids and triacylglycerols (Elam, *et al.*, 2001).

Medica 16 (β,β'-tetra-methyl hexadecanoic acid) is an ATP-citrate lyase inhibitor that limits acetyl-CoA supply to acetyl-CoA carboxylase thus inhibiting fatty acid synthesis (Goodridge, 1973). It is a long-chain fatty acyl analogue developed as a hypolipidemic and antiobesity compound (Bar-Tana, *et al.*, 1989). Treatment of JCR:LA-cp rats with Medica 16 reduces plasma lipid levels, hyperinsulinemia and plasma triacylglycerols to levels similar to the

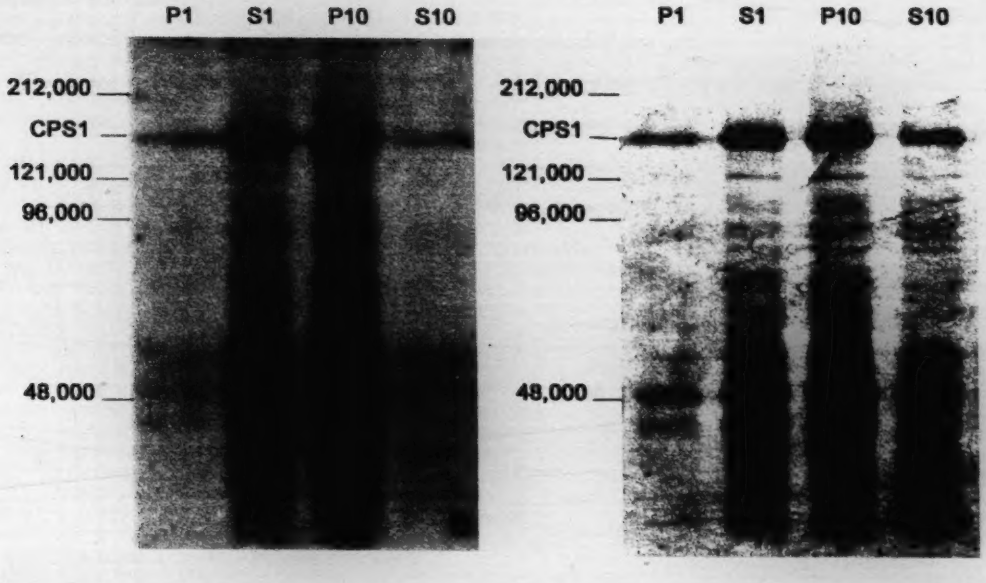


Figure 3.7 Mitochondrial fatty acylation is a proccess that occurs in tissue cultured cells

Freshly isolated rat hepatocytes were incubated with 100 μCi of [^{125}I]iodopalmitate for 4 h and then subjected to cell fractionation as described in "Materials and Methods". Aliquots of all fractions were loaded onto a 10% SDS-PAGE. The gel was stained with Coomassie Blue and exposed onto a phosphorimager cassette. A) Autoradiogram. B) Coomassie Blue staining.

lean counterparts (Russell, *et al.*, 1998). It has been shown that Medica 16 reduces acetyl-CoA carboxylase activity to lean rat levels (Atkinson, *et al.*, 2002).

Compound S (L-2-({2-methoxy-2-[3-(trifluoromethyl)phenyl]ethyl}amino) ethyl-4-2-{[2-(9H-9-fluorylenyl)phenyl]ethyl}bencene) is a compound developed for the oral treatment of type II diabetes. It decreases the expression of two key gluconeogenic enzymes, phosphoenolpyruvate carboxykinase and glucose 6-phosphatase (Russell, *et al.*, 2000). In contrast, it stimulates the expression of enzymes involved in glycolysis and lipogenesis such as acetyl-CoA carboxylase and acyl-CoA synthetase. Moreover, compound S decreases the expression of proteins that regulate the mitochondrial rate of oxidation of long-chain fatty acid (e.g. carnitine palmitoyltransferase 1) and ketone body production (hydroxyl-methyl-glutaryl-CoA synthase) and acetyl-CoA synthetase. JCR:LA-cp rats treated with compound S show a significant decrease in food intake and plasma insulin levels.

As it can be seen in figure 3.8 A, JCR:LA-cp rats show a decrease in CPS 1 activity. Interestingly, as mentioned above, treatment of JCR:LA-cp rats with Medica 16, an inhibitor of fatty acid synthesis, increases CPS 1 activity. On the other hand treatment of JCR:LA-cp rats with compound S, an insulin sensitizing compound, did not show any effect on CPS 1 activity when compared to obese rats.

Contrary to what can be observed in regards to the activity, CPS 1 expression is increased in JCR:LA-cp rats. This seems to be a compensatory effect between activity and expression (Figure 3.8, B).

It is fascinating that in a model of obesity (JCR:LA-cp rats) where hepatic fatty acid levels are increased, CPS 1 activity is reduced when compared to lean counterparts. Furthermore, when these obese rats are treated with Medica 16, a compound that inhibits fatty acid synthesis, CPS 1 specific activity is highly increased even to higher levels than lean rats. This implies that CPS 1 has a certain level of inhibition in lean animals.

In regards to compound S, little is known on its mechanism of action. However, it is known that it reduces the expression of two genes encoding the regulatory proteins of hepatic long-chain fatty acid oxidation, carnitine palmitoyltransferase 1 and mitochondrial hydroxyl-methyl-glutaryl-CoA synthase. This suggests that compound S may decrease the rate of hepatic fatty acid oxidation. Yet, CPS 1 activity and expression are not altered in obese rats treated with this compound and unfortunately, fatty acid levels were not determined under these conditions.

In summary, the *in vitro* studies on CPS 1 inhibition by palmitoyl-CoA correlate with the data obtain in the *in vivo* studies using obese and lean animals. The fact that both MMSDH and CPS 1 are involved in amino acid catabolism, are fatty-acylated and inhibited by physiological concentrations of

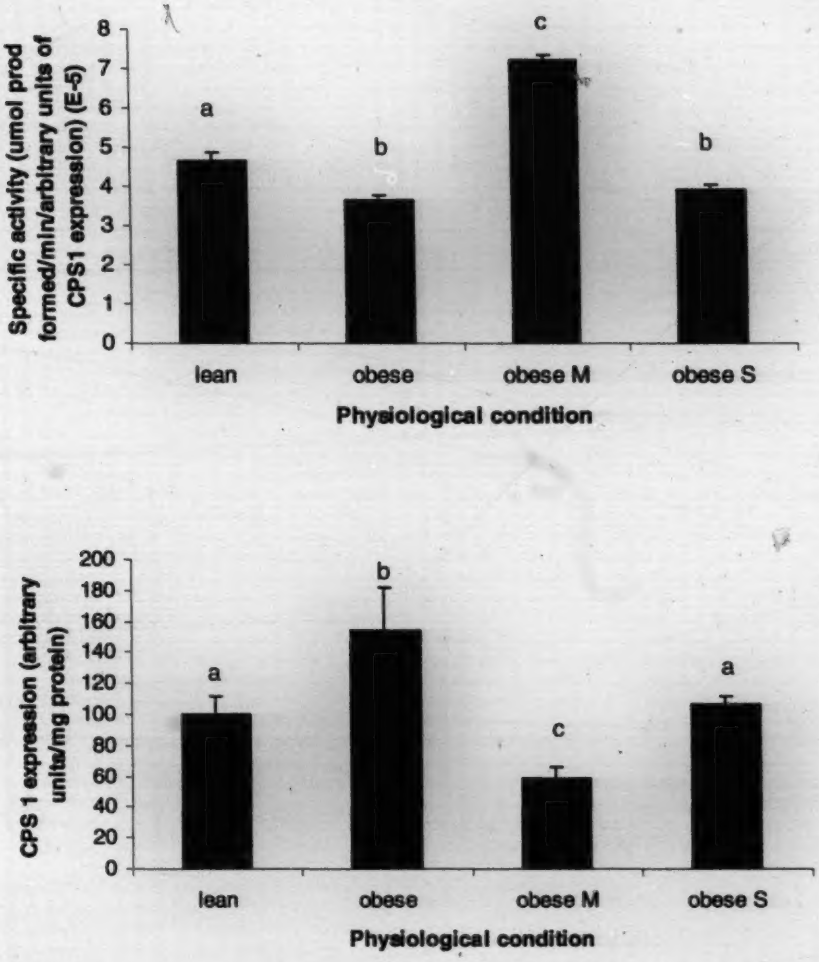


Figure 3.8 CPS 1 activity and expression under different physiological conditions.

Obese rats were treated with either compound M (inhibitor of fatty acid synthesis) or S (sensitizes cells towards insulin). Mitochondria were purified from all groups as described in "Materials and Methods", and 50 µg of protein obtained from solubilized mitochondria were assay for CPS 1 activity (A) or western blot analysis was performed with 1 µg of total protein (B). Different letters show differences with $p<0.05$ between all groups (one way ANOVA followed by Tukey test). N=6.

palmitoyl-CoA further reinforces the possibility of a metabolic cross-talk between amino acid and fatty acid catabolic pathways.

Whether the palmitoylation site on CPS 1 is on the active site, as it was the case for MMSDH, or not will be the subject of study of the next chapter.

CHAPTER 4

Development of recombinant Carbamoyl Phosphate Synthetase 1 expression systems

4.1 Overview

Since FSBA protected CPS 1 activity against palmitoyl-CoA inhibition and reduced CPS 1 fatty acylation levels, it was likely that active site cysteine(s) 1327, 1337 or both were the acceptor(s) of the palmitoylation. Consequently, we decided to perform site-directed mutagenesis to change these cysteine residues to serines. Acylation tests of these mutants would confirm whether these cysteines were in fact the residues that are palmitoylated.

In order to investigate the fatty acylation of these mutants, we needed a recombinant expression system. We tried to express CPS 1 constructs in McArdle RH-7777 cells, COS-7 cells, *Y. lipolytica* and in bacteria. Unfortunately, none of the expression systems allowed the expression of CPS 1 in such extent to measure its acylation.

The expression systems investigated are briefly described below.

4.2 Expression of WT and CPS1 active-site mutants in McArdle RH-7777 cells to generate a stable cell line.

We first tried to generate stable cell lines expressing the above mentioned CPS 1 constructs from the pCMV5 plasmid. In order to do this, we expressed these constructs in the rat hepatoma cell line McArdle RH-7777. We chose this cell line because it provides the most conserved environment for CPS 1. This cell line does not express CPS 1 endogenously.

4.2.1 Problems and solutions

We selected 100 colonies that were expressing our constructs. As can be seen in Figure 4.1, all the CPS 1 proteins were expressed in *McArdle RH-7777* as judged by western blot analysis. However, after storage, freezing and thawing and multiple passages, the expression of all constructs was lost. We repeated the screen for the identification of cell lines stably expressing the various CPS 1s, but to no avail. Consequently we decided to continue our studies using another expression system: transient transfection in COS-7 cells.

4.3 Expression of WT and CPS1 active-site mutants in COS-7 cells

COS cells are a stable cell line of African green monkey kidney cells that carry integrated copies of the SV40 genome and constitutively express biochemically active, SV40-encoded large T antigen. Because large T antigen is the only virally encoded product required to activate the SV40 origin of DNA replication, COS cells are able to support the replication of any plasmid that contains an intact SV40 origin (e.g., pCMV5 plasmid). Transfection of COS cells with recombinant plasmids containing an SV40 origin and a functional transcription unit leads to efficient amplification of the transfecting DNA and an enhanced level of transient expression of the cloned DNA fragment. COS-7 cells contain SV40 DNA sequences integrated at several sites in the genome (Rio, *et al.*, 1985).

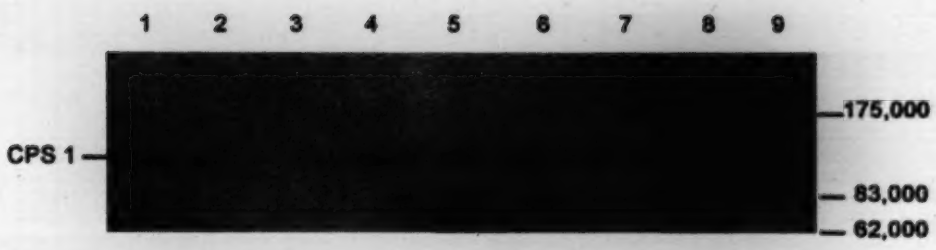


Figure 4.1 Expression of WT and various CPS1 active-site mutants in *McArdle RH-7777* clones

Typical results of a western blot showing the expression of different constructs for CPS 1. Lane1: 100 ng purified CPS 1; Lane 2: 1337 mutant (colony #11); Lane 3: 1337 mutant (colony #7); Lane 4: Full length CPS 1 (colony #7); Lane 5: 1337 mutant (colony #2); Lane 6: 1327/1337 (colony #8); Lane 7: 1337 mutant (colony #12); Lane 8: 1327 mutant (colony #12); Lane 9: pCMV5 (colony #7). All the lanes were loaded with 10 μg of purified mitochondria onto an 8% SDS-PAGE.

All the constructs were expressed in COS-7 cells as determined by western blot, but before performing the acylation test we needed to make sure that the constructs were properly folded. One way to test this is to perform solubility studies and partial trypsinization assays. Solubility studies allow us to make sure that the mutation does not produce an aggregated protein, while partial trypsinization studies are based on the assumption that only the residues that are more exposed are going to be cut in a short period of time. If the proteins are similarly folded, they will expose the same residues and thus, the trypsinization pattern will not vary between constructs. As can be seen in Figure 4.2, the solubility and the partial proteolytic rate did not differ significantly between the various CPS 1s. Once these controls were done, the acylation assay was carried out.

4.3.1 Problems and solutions

Although CPS 1 expression, as analyzed by western blot, showed a very strong signal, fatty acylation levels were too low to be detected by autoradiography. Therefore, we decided to perform these studies in yeast so as to increase the yield of protein expression and be able to perform the acylation test.

4.4 Expression of WT and CPS1 active-site mutants in *Y. lipolytica*

In order to test if CPS 1 could be expressed in yeast, we subcloned the wild type CPS 1 and the double mutant into pTC3, to be expressed in *Y.*

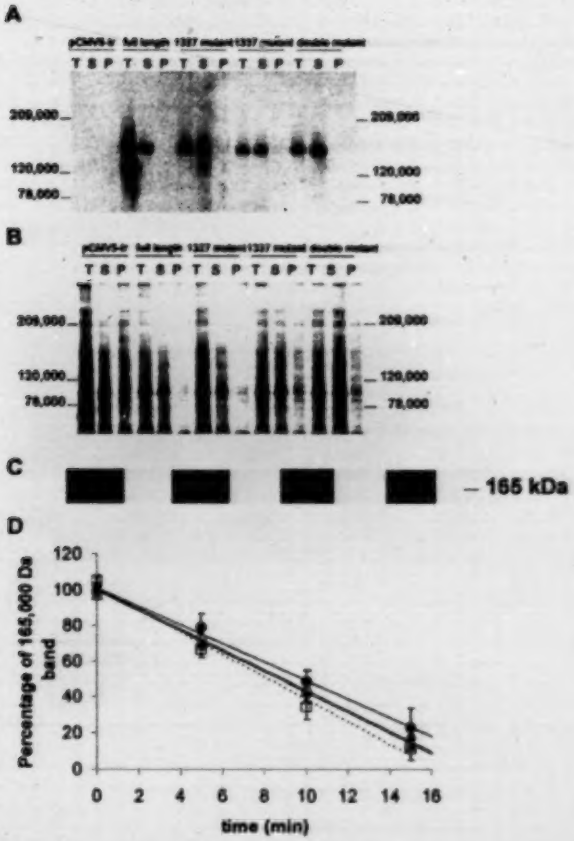


Figure 4.2 CPS 1 mutants are expressed as soluble proteins in COS-7 cells and are similarly folded

All CPS 1 constructs were transiently transfected into COS-7 cells. Mitochondria from all the constructs were purified and solubilized as described in "Materials and Methods". Western blot showing a typical solubility experiment (N=5)*(A)*, and India ink staining of PVDF membrane *(B)* T: total sample before solubilization, S: supernatant of solubilized mitochondria, P: pellet of solubilized mitochondria. Typical western blot signal for each time *(C)*. Graph showing the trypsinization results of all CPS 1 constructs (N=6). WT (•), C1327S (Δ), C1337S (□), C1327,1337S (♦) *(D)*. Slopes showed not to be significantly different by Anova Test $p<0.05$.

lipolytica. *Y. lipolytica* was the yeast of our choice since it is one of the most extensively studied yeasts, is not considered as pathogenic as other ascomycetous yeasts and because it expresses proteins that are most conserved to mammals.

4.4.1 Problems and solutions

At first, in order to verify if CPS 1 was expressed in *Y. lipolytica*, we lysed the yeasts with glass beads and performed a western blot to detect CPS 1. As can be observed in Figure 4.3, CPS 1 was expressed in this system, but there were too many total proteins to obtain a clear acylation pattern. Subsequently we decided to purify the mitochondria prior to Western blot analysis. The first step when fractionating yeast is to disrupt the cell wall to obtain spheroplasts. We obtained spheroplasts using a variety of methods: zymolase, lyticase, French press and β 1,3 endoglucanase. However, during mitochondrial purification, CPS 1 showed a very rapid degradation rate. As a consequence of this, there was not enough CPS 1 in the mitochondria so as to be detected by autoradiography. After this, we decided to try the expression of CPS 1 in bacteria.

4.5 Expression of WT and CPS1 active-site mutants in bacteria

In a first attempt, all CPS 1 constructs were subcloned into the bacterial expression vectors pET19b and pET12c and the bacterial strains BL21(DE)3 and AD494(DE)3 were transformed. CPS 1 expression was induced with

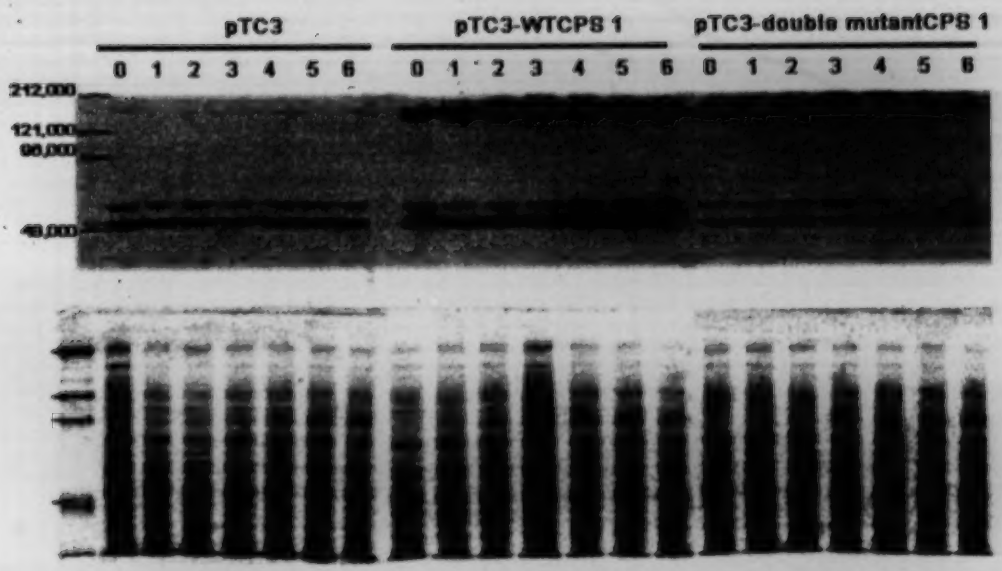


Figure 4.3 Expression of WT and double mutant CPS 1 in *Y. lipolytica*.
Western blot analysis of expression of WT and double mutant CPS 1 constructs and the hours of induction in oleic acid *(A)*. Coomassie blue staining of the PVDF membranes *(B)*.

IPTG. In all cases CPS 1 was localized to inclusion bodies and we could not get any amount of CPS 1 in a soluble form (data not shown). As a result, we decided to co-express CPS 1 with the GROEL/GROES chaperone system so as to increase the possibility of obtaining it in a soluble form. However, CPS 1 could never be expressed as a soluble protein, even in presence of the GROEL/GROES chaperone system.

4.5.1 Problems and solutions

We later found out that bacteria can rarely express properly folded proteins with masses higher than 60 kDa (CPS 1 is 165 kDa). Since all the expression systems failed, we decided to try to find the acylated cysteine residue in CPS 1 using mass spectrometry analysis of purified CPS 1.

4.6 Analysis of CPS 1 palmitoylation by mass spectrometry

In general terms, palmitoylation sites of either small synthetic peptides (Liang, *et al.*, 2002; Liang, *et al.*, 2004) or small proteins (Bizzozero, *et al.*, 2002; Trester-Zedlitz, *et al.*, 2005; Yang, *et al.*, 2005) have been detected using mass spectrometry analysis. However, since all the expression systems used failed we decided to perform mass spectrometry analysis on CPS 1. With this aim in mind, purified CPS 1 was treated or not with palmitoyl-CoA and subjected to mass spectrometry analysis.

4.6.1 Problems and solutions

Upon complete tryptic digestion CPS 1 gave rise to several peptides and we could never find a peptide with a putative additional mass of 238 Da. between the acylated and non-acylated samples. Overall, grater than 95% recovery of CPS 1 peptides was observed, but several peptides corresponding to the active site were missing. In addition, the acylated sample showed a greasy spot on the metallic surface where the TOF (time of flight) was performed (personal communication), suggesting that many peptides, perhaps including the acylated peptides, did not fly in the MALDI-TOF apparatus and thus, could not be detected. In a final attempt to detect the fatty acylation residues in CPS 1, we carried out limited proteolysis.

4.7 Mapping CPS 1 palmitoylation site by limited proteolysis

It is known that proteolytic cleavage of CPS 1 with trypsin gives rise to two fragments of 87,000 and 62,000 Da (Powers-Lee and Corina, 1986; Marshall and Fahien, 1988). In addittion, elastase is known to cleave CPS 1 at residues 417 and 1318, thus obtaining final fragments of 40,000 and 100,000 Da (Powers-Lee and Corina, 1986; Marshall and Fahien, 1988) (Figure 4.4). With the idea of narrowing our search for the palmitoylation site on CPS1, purified CPS 1 was treated with [^{125}I]iodopalmitoyl-CoA and subjected to limited proteolysis using trypsin and elastase. As can be observed in Figure 4.4, the palmitoylation fragment was determined to be between amino acids 417-1283.

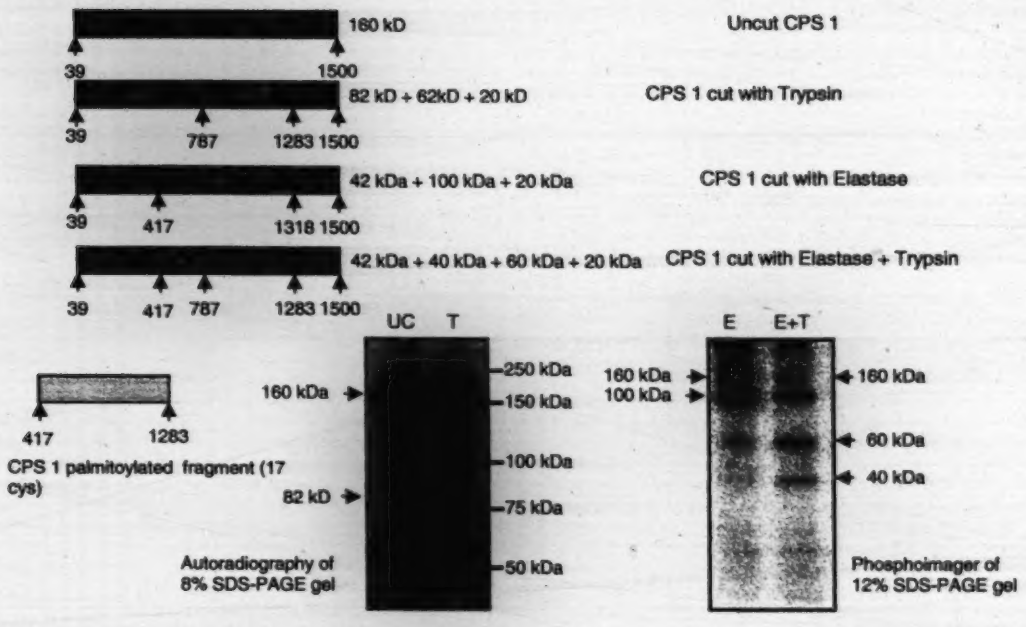


Figure 4.4 The CPS 1 palmitoylation fragment was determined to be between amino acids 417- 1283.

CPS 1 (6 μg) was incubated with [^{125}I]iodopalmitoyl-CoA for 30 min at 25°C was further incubated with either elastase or trypsin for another 30 min at 25°C. The reactions were stopped by addition of elastatinal (for elastase) or leupeptin (for trypsin). For serial digestions, the first partial digestion was stopped with the specific protease inhibitor and then the second partial digestion took place under the same conditions. Samples were then subjected to SDS-PAGE, stained, destained, dried and exposed onto a film or phosphorimager cassette.

4.7.1 Problems

The palmitoylation fragment obtained by limited proteolysis analysis contains 17 cysteine residues. Since there is not any consensus sequence for mitochondrial palmitoylated proteins, palmitoylation could occur at any of them and we could not identify the acylated cysteine in CPS 1. However, fragment 417-1283 contains the AGA binding site (residues 625-630) that facilitates the binding of ATP (residues 631-638) (Figure 4.5). Both binding sites are located in the B domain of CPS 1. The B domain contains cysteine residues at positions 516, 600, 644, 648, 697, 711 and 761; all very close to the AGA and ATP binding sites. The fact that FSBA interacts with cysteines 1327 and 1337 which are located in the ATP binding site, but on the D domain can explain the inhibition obtained at the acylation level in figure 3.4 using AGA in combination with either ATP or FSBA.

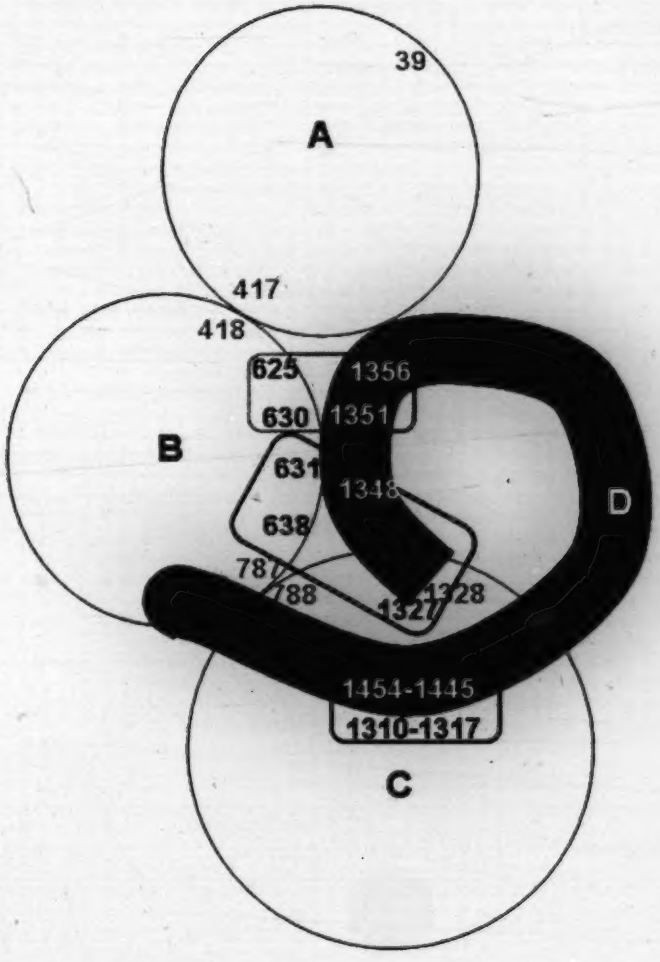


Figure 4.5 Scheme of ATP and AGA binding sites on CPS 1.

The AGA site has components of the B (residues 625-630) and D (residues 1351-1356) domain. The ATP_b site is centered on domain B (residues 631-638) with components from domain D (residues 1327-1348). The ATP_c site is centered on domain C (residues 1310-1317) with components from domain D (1445-1454). Amino acids that define domain boundaries are shown in red. (Adapted from Powers-Lee & McCudden, 1996).

CHAPTER 5

Development of a novel non-radioactive labeling method for the detection and isolation of mitochondrial palmitoylated proteins

5.1 Overview

Although fatty acylation may play a key role in the regulation of mitochondrial processes, no efficient and convenient method for detection, quantification and proteomic analysis of these modifications exist to date. The traditional methodology used to study fatty acylation of proteins involves the use of radioactive fatty acid analogues (e.g. [^{3}H]- or [^{14}C]- palmitate or [^{125}I]-iodopalmitate). These radioactive fatty acids have many disadvantages. First, tritiated fatty acids which typically have their labeled hydrogens attached to carbons in positions 9 and 10 can be shortened by partial β-oxidation. In some cases, longer incubation times can result in radioactivity incorporated into the amino acid pool of nascent proteins, resulting in metabolic labeling of proteins. In addition, detection of acylated proteins having incorporated tritiated fatty acids usually needs long exposure times (1-3 months) to obtain a quantitative signal. Peseckis and coworkers (Peseckis, *et al.*, 1993; Berthiaume, *et al.*, 1995) developed a series of [^{125}I]iodofatty acid analogues to study fatty acylation. These fatty acid analogues were designed to minimize steric differences with the native fatty acid, and thus the iodine was introduced in the omega position of the linear saturated fatty acid chain (Peseckis, *et al.*, 1993). The use of these [ω-^{125}I]iodofatty acid analogues to study fatty acylation had on one hand the advantage of reducing exposure times when compared to [^{3}H]fatty acid analogues and that they could not be converted into radioactive

amino acids, but on the other hand they also have the disadvantage of requiring the handling of a high-energy radioisotope.

In order to rapidly detect and isolate fatty acylated proteins we sought to develop a non-radioactive methodology that would allow us to specifically add a series of chemical handles (e.g. biotin, Flag-tag, Myc-tag) to fatty acylated proteins. The methodology developed here involves the metabolic incorporation of synthetic azidofatty acid analogues and chemoselective derivatization of the azidofatty acylated proteins with a tagged triaryl phosphine capture reagent by a modified version of the Staudinger reaction, also known as Staudinger ligation (Saxon and Bertozzi, 2000; Kiick, *et al.*, 2002; Saxon, *et al.*, 2002; Kho, *et al.*, 2004).

The Staudinger ligation includes three steps: the attachment of the azidopalmitoyl-CoA onto proteins, the ligation of the modified protein via the azido moiety to a tagged triaryl phosphine capture reagent and finally the detection of the conjugated products (Figure 5.1). Azidopalmitoyl-CoA (N_3C_{14}-CoA) has been designed to be similar to the natural substrate in size and thus allow the natural reaction to proceed. Alkyl azides have been used in cells and animal studies without any significant toxicity (Prescher, *et al.*, 2004) and are even clinically approved drugs for use in humans (e.g., AZT treatment of AIDS). Furthermore, azidofatty acids have the advantage of being readily incorporated into long-chain fatty acyl-CoAs and proteins (Devadas, *et al.*, 1992), and are not toxic to cells and animals (Devadas, *et al.*, 1992).

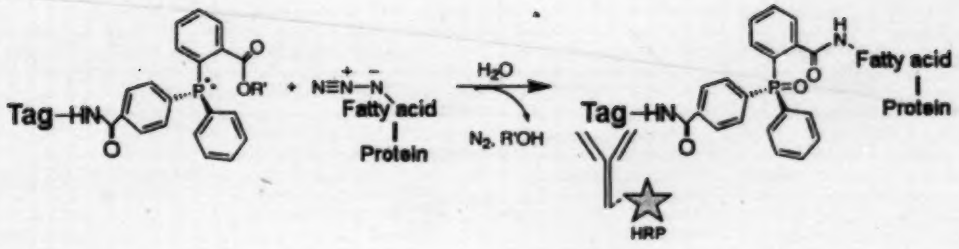


Figure 5.1 Schematic representation of the Staudinger ligation for the detection of palmitoylated proteins.

A tagged triaryl-phosphine capture reagent reacts specifically with the azido moiety of the fatty acid that is already attached onto proteins. The conjugated product can be separated, blotted and detected using appropriate anti-tag antibodies and ECL detection protocol (Materials and Methods section). In addition, the tagged acylated protein can be pulled down using avidin-agarose for the purpose of identifying these proteins.

The aim of this chapter is to show the advantages that this methodology offers as an approach for the detection and identification of mitochondrial fatty acylated proteins. Furthermore, this new technology could be used in the isolation of fatty acylated proteins by performing pull down experiments. Identification of mitochondrial fatty acylated proteins is the first step to understand the role of this important posttranslational modification on mitochondrial metabolic pathways.

5.2 A similar profile of mitochondrial palmitoylated proteins can be detected either by using azido- or radiolabeled-fatty acids

In order to investigate if the azidofatty acid behaves in the same manner as the radiolabeled analogues, we labeled lysed rat liver mitochondria with either azidopalmitoyl-CoA followed by incubation with Myc-phosphine capture reagent, or with [^{125}I]iodopalmitoyl-CoA. Azide incorporation was visualized by western blot using an anti-Myc antibody and ECL protocol, and radiolabel incorporation into proteins was visualized by autoradiography. We found similar profiles of labeled proteins became visible regardless of the method used. Furthermore, the profile of proteins found to be fatty acylated by this new method is similar to the profile obtained by labeling primary culture of hepatocytes with [^{125}I]iodopalmitate (page 85). This demonstrates that the *in vitro* labeling system is a valid method to study fatty acylation and that correlates to what is observed *in vivo*. In addition, CPS 1 can be readily

detected by both methods, showing that the azido moiety does not alter the fatty acylation pattern.

Although the distribution of fatty acylated proteins were similar regardless of the methodology employed, the acylation levels of bands of similar apparent molecular weight varied in some cases, being higher always when azidofatty acids were used (Figure 5.2). This was due to the higher sensitivity of this method when compared to the traditional use of radioisotopes and the detection method used in each case. Whereas the signal obtained by the phosphor screen is directly proportional to the number of molecules of ^{125}I, the signal detected by ECL is exponentially amplified from each molecule of Myc.

In comparison to the problematic long exposure time (1-3 months) required for detecting [^{3}H]fatty acid incorporation into fatty acylated proteins, our method represents a scientific and technical breakthrough, since our typical exposure times are only 1-3 seconds. This represents over 2.6 million-fold signal amplification versus fluorography/autoradiography (1-3 months versus 1-3 seconds).

5.3 Azidopalmitoyl-CoA is efficiently incorporated onto proteins *in vitro*

In order to establish the efficiency of protein palmitoylation labeling *in vitro*, lysed mitochondria and purified CPS 1 were labeled with or without azidopalmitoyl-CoA, followed by incubation in presence or absence of Myc-phosphine capture reagent. Azide incorporation was visualized by western blot

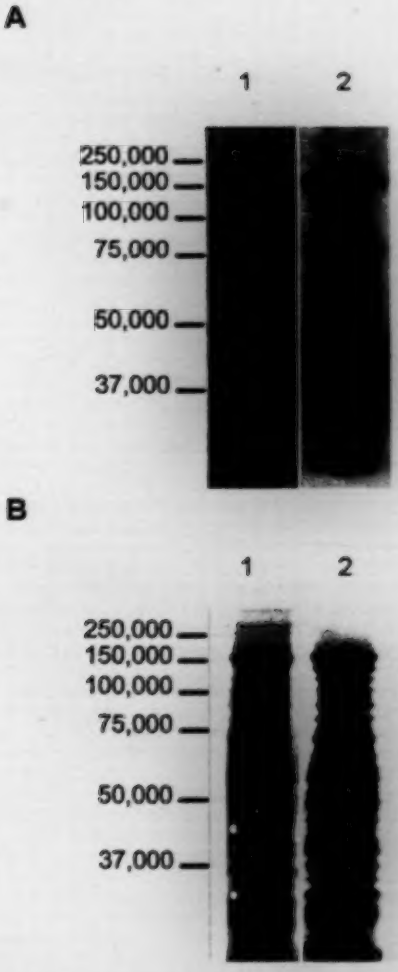


Figure 5.2 Detection of mitochondrial palmitoylated proteins by different methods yields similar results.

Lysed mitochondria (10 µg) were labeled with either azidopalmitoyl-CoA or [^{125}I] palmitoyl-CoA analogues as described under "Materials and Methods". Western blot for azidofatty acid detection (lane 1) or autoradiogram (lane 2) of the corresponding SDS-PAGE *(A)*. India ink staining *(B)*. These experiments were performed on different experiments with different gel percentages, being 10% for lane 1 and 8% for lane 2.

using an anti-Myc antibody. We found that the Myc-label was only found when the samples were incubated in the presence of both azidopalmitoyl-CoA and myc-phosphine capture reagent (Figure 5.3), indicating that the labeling by this method is highly specific. There was no background signal when only the azidofatty acid or the Myc-phosphine capture reagent was used.

5.4 Hydroxylamine treatment removes the azido-label from proteins

To study the chemical nature of the bond linking the azidofatty acid to proteins, the samples were incubated with 1 M hydroxylamine, pH 7.0, for 16 h after being treated with the capture reagent. This treatment is known to cleave thioester but not oxyester bonds (Magee, *et al.*, 1984; Schmidt and Lambrecht, 1985; Ross and Braun, 1988). As shown in Figure 5.4 panel B, we included CPS 1 (a known palmitoylated protein) (Corvi, *et al.*, 2001) in all the experiments as a positive control. Neutral hydroxylamine treatment removed virtually all the label (98.25 % +/- 0.36 (n=6)) when compared to 1 M Tris-HCl, pH 7.0 control treatment (Figure 5.4 A and C). This result indicates that the azide moiety does not affect the type of bond by which the palmitate modifies the proteins and thus, the azidofatty acid behaves as the naturally occurring fatty acid, binding to proteins via a hydroxylamine sensitive thioester bond.

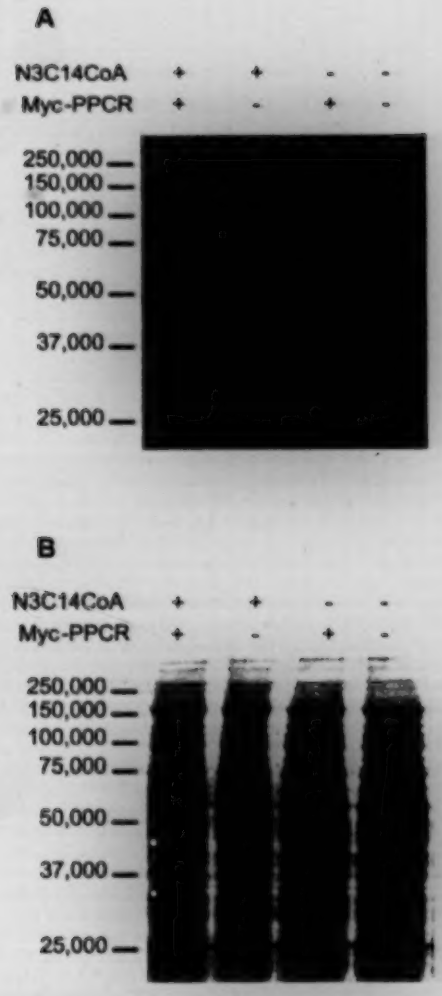


Figure 5.3 Efficiency of mitochondrial protein palmitoylation labeling *in vitro*. Lysed mitochondria (10 µg) were labeled with 50 µM of azidopalmitoyl-CoA analogue as described under "Materials and Methods". Azidofatty acylated protein detection by western blot using an anti-Myc antibody and ECL protocol *(A)*. India ink staining of the corresponding membrane *(B)*.

5.5 Pre-treatment of mitochondria and CPS 1 with N-ethylmaleimide strongly reduces the incorporation of azidopalmitoyl-CoA

In order to test if the acceptor residue on the protein was a cysteine, as is generally the case for palmitoylation, we pre-treated the samples with N-ethylmaleimide. This compound is known to alkylate the sulfhydryl group of cysteine residues, blocking the potential covalent acylation of these proteins. We observed an inhibition of azidopalmitate incorporation into proteins pre-treated with N-ethylmaleimide (Figure 5.5). The extent of the inhibition was 99.32 % +/- 0.81 (n=6). Furthermore, CPS 1 labeling is completely prevented by pre-treatment with N-ethylmaleimide, which is in accordance with our previous results when using [^{125}I]iodopalmitoyl-CoA (Corvi, *et al.*, 2001). It was previously shown using radiolabeled palmitoyl-CoA (Soltys, 1999; Corvi, *et al.*, 2001), that CPS 1 acylation is likely occurring through a thioester bond because neutral hydroxylamine treatment removed the fatty acid from CPS 1. Furthermore, the protein acceptor residue is a cysteine since pre-treatment of CPS 1 with N-ethylmaleimide prevents fatty acylation. These experiments show that the azide-modified protein maintains the major characteristics of naturally occurring palmitoylated proteins since neutral hydroxylamine treatment removes the fatty acid from proteins (Figure 5.4) and pre-treatment of mitochondrial proteins or CPS 1 with N-ethylmaleimide prevents the incorporation of the azido-fatty acid (Figure 5.5). Overall, our results confirm

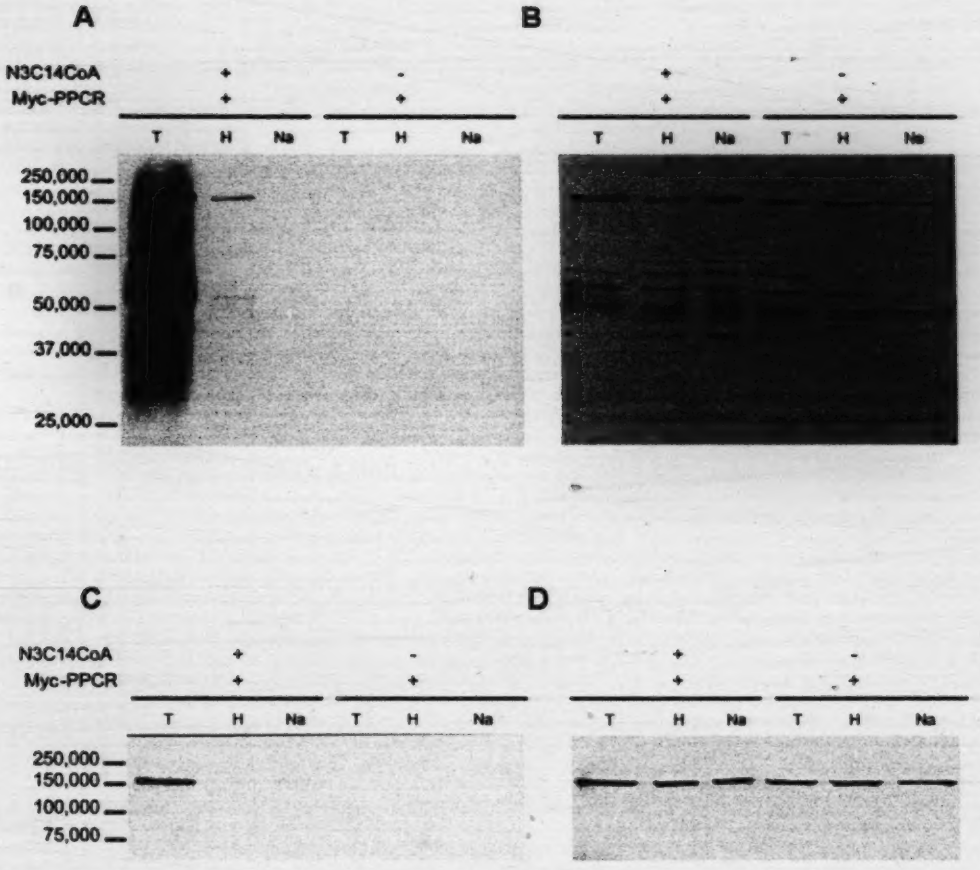


Figure 5.4 Azidopalmitate is bound to proteins via a hydroxylamine-sensitive thioester bond.

Lysed mitochondria (10 μg) or purified CPS 1 (1 μg) were labeled with 50 μl of the azidopalmitoyl-CoA analog and then treated either with 1M NH_2OH, 1M Tris or 0.2 N NaOH as described under "Materials and Methods". Azidofatty acylated protein detection of lysed mitochondria by western blot using an anti-Myc antibody and ECL protocol *(A)*. Corresponding India ink staining of the PVDF membrane *(B)*. Detection of azidofatty acylated CPS 1 by western blot *(C)*. Corresponding India ink staining of the membrane*(D)*.

that the azide moiety does not affect the binding of palmitate to the proteins and thus, the azidofatty acid behaves as the naturally occurring fatty acid modifying cysteine residues of proteins.

5.6 Different tagged phosphine capture reagents can be used to detect azido-modified proteins

In order to test if the use of different phosphine capture reagents yields the same profile of palmitoylated proteins, lysed mitochondria were labeled with or without azidopalmitoyl-CoA, followed by incubation with either fluoresceine- (Figure 5.6, lane 1), Flag- (Figure 5.6, lane 2) or Myc-phosphine capture reagent (Figure 5.6, lane 3). Azidofatty acid incorporation was visualized by western blot using the appropriate anti-tag antibody (Flag or Myc) or by fluorescence (fluorescein). We found a similarity in the profiles of palmitoylated proteins when the capture reagent used was Flag- or Myc-tagged. The bands showing the strongest signal in these western blots also appear on the fluorescein lane with much less intensity. This is due to the detection system employed to detect the signal in both cases. When the Flag- or Myc-PPCRs are used the detection is by ECL and thus, the original signal is exponentially amplified. However, the signal obtained by the phosphorimager used in the fluorescent scan mode with the fluorescein-PPCR labeled acylated proteins is linear compared to the original signal emitted. Furthermore, the profile of proteins here obtained with Flag- or Myc-PPCR is the typical profile obtained

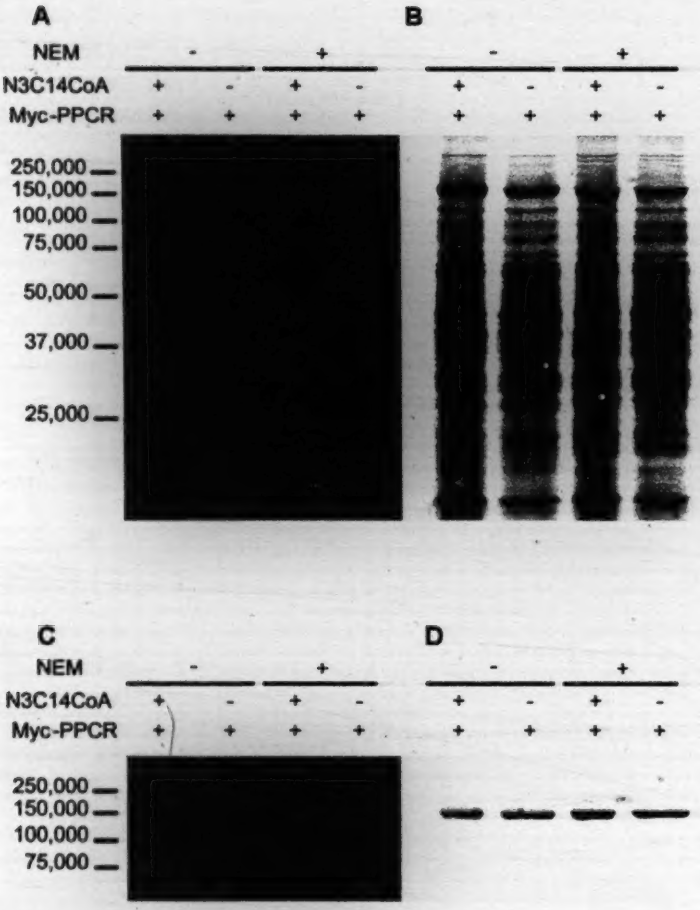


Figure 5.5 Cysteine residues are the acceptor residues on the proteins of the azidofatty acid.

Lysed mitochondria (10 µg) or purified CPS 1 (1 µg) were pre-treated with 10 mM N-ethylmaleimide followed by incubation with 50 µM azidopalmitoyl-CoA and the Myc-tagged phosphine capture reagent, as described under "Materials and Methods". Azidofatty acid detection of lysed mitochondria by western blot *(A)*. Corresponding India ink staining of PDVF membrane *(B)*. Azidofatty acid detection of purified CPS 1 by using an anti-Myc antibody by western blot *(C)*. Corresponding India ink staining of PVDF membrane *(D)*.

for mitochondrial fatty acylated proteins (Corvi, *et al.*, 2001).

5.7 MS analysis of azidopalmitoylated proteins identifies palmitoylated proteins.

With the aim of identifying new mitochondrial palmitoylated proteins, lysed mitochondrial proteins were labeled with or without azidopalmitoyl-CoA and Myc-phosphine capture reagent. The proteins were loaded on a 10% SDS gel for either Western Blot detection (10 μg) or Coomassie Blue staining (90 μg). Azide incorporation was visualized by western blot using an anti-Myc antibody and three bands showing ample resolution were selected from the western blot for identification. The equivalent bands from the Coomassie Blue gel were cut out and identified using mass spectrometry (MS) (Figure 5.7). The MS analysis of the three bands selected showed that CPS 1, pyruvate carboxylase (PC) and aspartate aminotransferase (AAT) as putative palmitoylated proteins.

This novel methodology allowed us to identify for the first time more putative mitochondrial palmitoylated proteins and also to confirm already known palmitoylated proteins. First we found, as expected, CPS 1. Another putative fatty acylated protein identified by this method for the first time was pyruvate carboxylase (PC) –E.C 6.4.1.1. PC is a 130 kDa mitochondrial protein involved in the irreversible carboxylation of pyruvate to form oxaloacetate, which may be ultilized in the synthesis of fat, glucose and aminoacids. It is interesting to note that aspartate aminotransferase (AAT; E.C. 2.6.1.1) was also detected as another putative palmitoylated protein. This

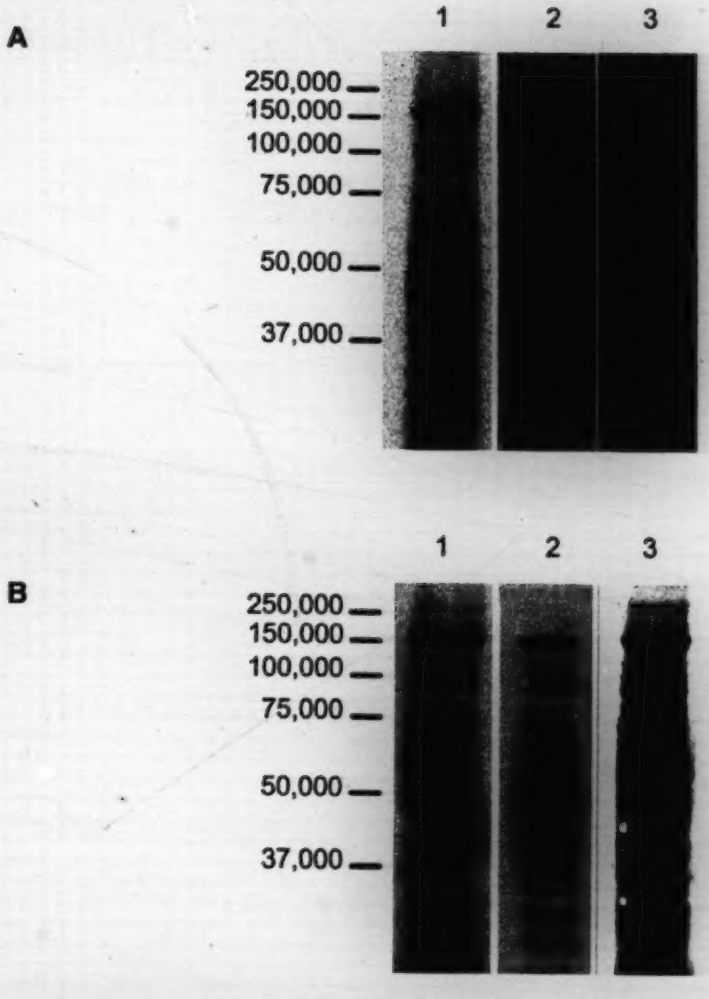


Figure 5.6 Profile of mitochondrial palmitoylated proteins detected by different tagged phosphine capture reagents.

Lysed mitochondria (10 µg) were labeled with azidopalmitoyl-CoA and then incubated with either fluorescein- (lane 1), Flag- (lane 2) or Myc-(lane 3) tagged phosphine capture reagent, as described under "Materials and Methods". A) Azidofatty acid detection of lysed mitochondria by western blot or fluorescence scanning of SDS-PAGE gel. B) Corresponding Coomassie Blue staining.

47.5 kDa mitochondrial protein (Pol, *et al.*, 1989) catalyzes the formation of oxaloacetate and glutamate from oxoglutarate and aspartate. Although these putative palmitoylated proteins were sufficiently separated in the gel and the MS analysis showed exclusively the peptide masses corresponding to PC and AAT (without any trace of peptides corresponding to other proteins), we still need to perform experiments leading to the confirmation of the covalent attachment of palmitate to these proteins. Besides, how fatty acylation influence these enzymes will require further experimentation.

This new methodology can be used extensively to study fatty acylation in other organelles.

In this chapter we showed that this method is highly specific, sensitive and that azidofatty acids share all the characteristics of the naturally occurring palmitate. We have no doubt that this methodology will help to identify which mitochondrial proteins are modified by palmitate and how this modification affects mitochondrial metabolism.

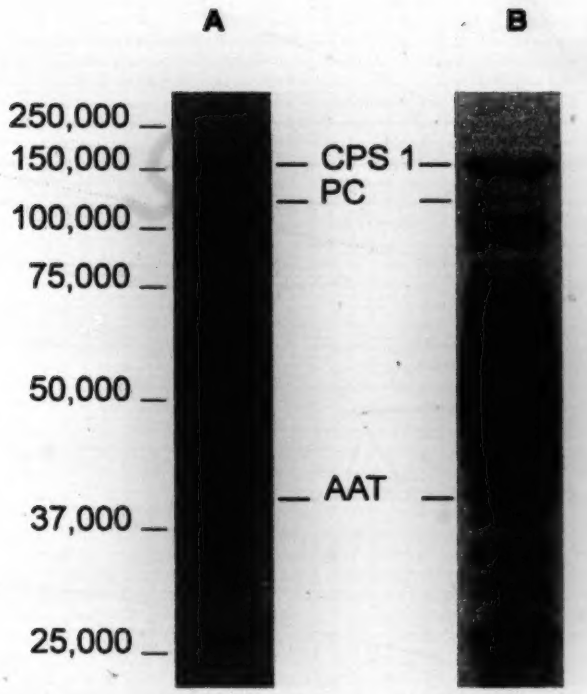


Figure 5.7 Western blot showing mitochondrial proteins subjected to MS analysis.

100 µg of lysed mitochondrial proteins were labeled with azidopalmitoyl-CoA, followed by incubation with Myc-phosphine capture reagent as described in the "Materials and Methods" section. Azide incorporation was visualized by western blot (A) as described above and bands that were palmitoylated and sufficiently separated from the each other were cut out from the gel (B) and sent for MS analysis. The bands identified by MS analysis were carbamoyl phosphate synthetase 1 (CPS 1, as expected), pyruvate carboxylase (PC) and aspartate aminotransferase (AAT).

CHAPTER 6

DISCUSSION

6.1 Fatty acylation in mitochondria

Although fatty acylation is a universal feature of eukaryotic cells, little is known about this important posttranslational modification in mitochondria. Until now, there are only four reports in this area. Two studies on the topic of fatty acylation in mitochondria were performed on the fatty acylated mitochondrial protein MMSDH (Deichaite, *et al.*, 1993; Berthiaume, *et al.*, 1994) and in 1989, Stucki and coworkers studied the distribution of fatty acylated proteins in mitochondria and showed that fatty acylation mainly occurred in the mitochondrial matrix (Stucki, *et al.*, 1989). Corvi and coworkers (2001) also analyzed the fatty acylation patterns of mitochondria from different tissues confirming the distribution pattern found by Stucki and coworkers. In these studies, it was found that the number of fatty acylated proteins were highest in the liver and lowest in the brain, with each tissue having several specific labeled proteins (Corvi, *et al.*, 2001). Furthermore, Corvi and coworkers (2001) showed that different tissues had differential fatty acylation patterns due to either specific expression of proteins or to different metabolic requirements of the individual mitochondria.

It is important to note that the matrix and, to a lesser extent, the inner mitochondrial membrane were shown to contain all the fatty acylated proteins (Stucki, *et al.*, 1989; Corvi, *et al.*, 2001). Since these two submitochondrial compartments are important sites for the catabolism of substrates required for energy production, it is possible that fatty acylation may reflect a novel type of

metabolic regulation. In addition, since the activity of the matrix-associated proteins MMSDH and CPS 1 were found to be drastically reduced by fatty acylation, the idea of a metabolic cross-talk between fatty acid and amino acid oxidation is reinforced. However, this hypothesis needed to be tested further and as mentioned in Chapter 1, it is the main objective of the present thesis.

6.2 Carbamoyl Phosphate Synthetase 1

As was mentioned in Chapter 1, CPS1 is involved in the part of amino acid catabolism involved in the removal of ammonia. It catalyzes the first and rate limiting step of the urea cycle (Krebs, *et al.*, 1973; Ratner, 1973). On high-protein diets the carbon skeletons of the amino acids are oxidized for energy production, can be recycled for *de novo* amino acid synthesis or stored as fat and glycogen, but the ammonia must be excreted.

Ammonia is one of the most toxic compounds produced by the body. Elevated levels (hyperammonaemia) can cause symptoms of ammonia intoxication: tremors, slurred speech and blurred vision. At very high concentrations, ammonia causes irreversible brain damage, coma and death. It is therefore essential that ammonia be detoxified rapidly to urea by the liver. Short-term regulation of the cycle occurs principally at CPS 1 level and as such, CPS 1 proper regulation is of utmost importance for life.

6.2.1 Characterization of CPS 1 palmitoylation

In 1999, CPS 1 was identified as the second fatty acylated mitochondrial protein (Soltys, 1999). In addition, it was shown that CPS 1 was covalently modified on a cysteine residue with iodopalmitate via a thioester bond (Soltys, 1999). Palmitoylation decreased CPS 1 activity, although the mechanism of the inhibition was not studied (Soltys, 1999). We confirmed that palmitoylation drastically reduces CPS 1 activity in a time- and concentration-dependent manner (Figure 3.1)(Corvi, *et al.*, 2001), at concentrations of palmitoyl-CoA well within the physiological levels found in the mitochondrial matrix (less than 230 μM; (Faergeman and Knudsen, 1997)). Inhibition of CPS 1 by palmitoyl-CoA is believed to be specific and not due to detergent-like properties of long chain acyl-CoA molecules that could occur at concentrations above the critical micellar concentration. Critical micellar concentrations for palmitoyl-CoA have been established by a variety of methods to be no lower than 60–80 μM (Powell, *et al.*, 1981). These values are more than double that of our calculated *Ki* of 19 μM for palmitoyl-CoA inhibition of CPS 1. These results would predict that CPS1 should be almost completely inhibited under physiological conditions. Indeed, mitochondrial long-chain fatty acyl-CoA levels can be as high as 600 μM during starvation (Owen, *et al.*, 1969). Nonetheless, with CPS 1 concentration estimated to be 0.4-1.5 mM (Meijer, *et al.*, 1990), the concentration of long-chain acyl-CoAs in the mitochondria would only be sufficient to inhibit a large portion of CPS 1 activity, but

potentially not all of it. This observation could explain the partial but not total reduction in urea secretion observed during prolonged starvation (Owen, *et al.*, 1967; Owen, *et al.*, 1969; Cahill, 1976).

CPS 1 fatty acylation appeared to be irreversible in the presence of an excess of palmitoyl-CoA *in vitro* using highly purified enzyme (Figure 3.5). Once fatty acylated, CPS 1 could be targeted to the inner mitochondrial membrane and there play a role as a scaffold protein. Furthermore, CPS 1 is known to enhance the interaction between GDH-AAT even in the presence of the substrates of these enzymes (Fahien, *et al.*, 1984). CPS 1 is the most abundant mitochondrial protein in liver (Clarke, 1976; Lusty, 1978; Fahien and Kmiotek, 1979) and only a fraction of it is active (Raijman and Jones, 1976). But how a matrix associated protein such as CPS 1 can interact with the inner mitochondrial membrane bound AAT? One possibility is that, as is the case for some cytosolic palmitoylated proteins, addition of palmitate could direct CPS 1 to the inner mitochondrial membrane and thus, allow the interaction with AAT and GDH. This structural role for CPS 1 could explain why this protein is present in such high levels. Another interesting possibility is that an isoform of APT may exist in mitochondria and thus, CPS 1 palmitoylation would be a reversible modification *in vivo* and as such it would act as a "molecular switch". A fascinating question that arises from this hypothesis is what would be the fate of the released fatty acid. It is known that FAS are expressed only in the cytosol, so a mechanism should be in place for the free fatty acid to be

exported from the mitochondria to the cytosol. One possible mechanism to export free fatty acids from the mitochondria is by means of the uncoupling proteins (UCPs). UCPs are located in the inner mitochondrial membrane and are proposed to transport free fatty acids out of the mitochondria (Dulloo, *et al.*, 2001). Interestingly, UCP-2 is widely expressed and lipids stimulate it expression in hepatocytes (Armstrong and Towle, 2001). It has been proposed that UCP-2 could play a role in the regulation of metabolism and not in thermogenesis, as is the case for UCP-1 (Klingenspor, 2003). As such, UCP-2 could be responsible of removing palmitate from the mitochondria.

In order to obtain more information on the possible palmitate acceptor site/s on CPS1, we performed substrate protection assays and measured CPS 1 activity (Figure 3.3) and acylation levels (Figure 3.4). We found a correlation between fatty acyaltion levels and CPS1 activity inhibition. Furthermore, pre-incubation of CPS 1 with AGA+ATP led to almost complete protection against both CPS 1 inhibition and fatty acylation. These results suggested that the ATP_b binding site (ATP binding site that is exposed upon AGA binding) was likely to be the acceptor of the palmitate. This site is centered on CPS 1 B domain (residues 631-638) with residues 1327-1348 of the D domain (Powers-Lee and Corina, 1986). Since the 17 amino acid sequence surrounding cysteine 1327 of CPS 1 is similar to the sequence surrounding the acylated cysteine 319 on MMSDH (cysteine that is acylated in MMSDH), we decided to

investigate whether cysteines 1327 or 1337 on CPS 1 were the acceptors of the palmitate (Figure 6.1).

In order to do this, we performed site directed mutagenesis and expressed all the constructs in a variety of expression systems (Chapter 4). Unfortunately, none of the expression system tested (bacteria, yeast and mammalian cells) allowed sufficient expression of properly folded CPS 1 and mutants so as to detect acylation of the recombinant proteins. Since we could not express the recombinant CPS 1s, we assayed limited proteolysis of the fatty acylated native mature CPS 1 protein. Using this technique, the smaller acylated fragment obtained (aa 417-1283) contained 17 cysteines, all susceptible of being the acceptor residues. However, having in mind the results obtained in the substrate protection assays with AGA+MgATP or AGA+MgFSBA, it is more likely that the acceptor cysteine is localized close to the ATP_b binding domain. Alternatively, a conformational change could occur in CPS 1 in the presence of AGA+MgATP and AGA+MgFSBA that could prevent fatty acylation from taking place, even at a distal binding site.

6.2.2 CPS1 regulation under different physiological conditions

It is well known that the concentration of long-chain fatty acyl-CoAs fluctuate considerably under different physiological conditions. A 2-3 fold increase in the concentration of palmitoyl-CoA has been observed in fasted and diabetic animals (Bortz and Lynen, 1963; Tubbs and Garland, 1964; Woldegiorgis, *et al.*, 1985). This increase is mainly due to inhibition of

MMSDH: A G Q R C[319] - M A L S T - - A I L V G E

CPS 1: P I L R C[1327] E M A - S T G E V A C F G E

Figure 6.1 Alignment of the sequence surrounding the active site residue in bovine liver MMSDH with a potential fatty acylation site in CPS 1.

Amino acid identities are shown in red and amino acid substitutions in blue.

enzymes involved in the *de novo* synthesis of fatty acids by palmitoyl-CoA (Kawaguchi and Bloch, 1974; Hsu and Powell, 1975). Our results suggest that under physiological conditions where fatty acid levels are high, CPS 1 activity would be decreased. In order to test our hypothesis, CPS 1 activity and expression were studied in rats that showed elevated concentration of fatty acids on both plasma and liver (JCR:LA rats) (Figure 3.7). It was clear that CPS 1 activity was reduced in obese animals when compared to lean counterparts. Moreover, when these obese animals were treated with Medica 16 (a fatty acid synthesis inhibitor), CPS 1 activity was recovered to even higher levels than that of the lean rats, showing that fatty acids are involved in CPS 1 activity inhibition. Furthermore, these results suggest that even in lean rats, there is a certain level of CPS 1 inhibition perhaps via its acylation. Although urea secretion was not measured in these experiments, in prolonged starvation -a similar metabolic situation where fatty acids are high- urinary nitrogen secretion is reduced 60-80% (Owen, *et al.*, 1967; Owen, *et al.*, 1969; Cahill, 1976). Of interest, most of this reduction comes from a 75-95% reduction in urea synthesis (Owen, *et al.*, 1967; Owen, *et al.*, 1969; Cahill, 1976). Therefore, CPS1 inhibition by palmitoyl-CoA could account for part of the reduction observed in the urea cycle activity.

It would have been interesting to have determined protein fatty acylation levels in this rat model and compared them to lean rats. This could be performed by perfusing the livers with a solution containing azido fatty acids,

isolating the mitochondria followed by determination of fatty acylated proteins. In this manner we could have a piece of data on fatty acylation levels *in vivo*.

6.3 Cross-talk between different metabolic pathways

6.3.1 Cross-talk between amino acid catabolism and fatty acids

The enzyme aspartate aminotransferase [L-aspartate:2-oxoglutarate aminotransferase, E.C. 2.6.1.1, AAT] has been detected with the azido fatty acid analogues as a putative acylated protein. In liver, AAT binds to the inner mitochondrial membrane and must be transferred to lipids to become fully activated (Teller, *et al.*, 1990; Fahien and Teller, 1992). AAT can participate in a number of transient complexes with other enzymes such as glutamate dehydrogenase and CPS 1, that have the potential to influence metabolism (Fahien, *et al.*, 1977; Beeckmans and Kanarek, 1981; Fahien, *et al.*, 1988; Fahien, *et al.*, 1989; Beeckmans, *et al.*, 1990; Teller, *et al.*, 1990). The evidence for the formation of transient hetero-enzyme complexes comes, for the most part, from *in vitro* studies using purified enzymes and purified mitochondrial membranes from various tissues (Fahien, *et al.*, 1977; Beeckmans and Kanarek, 1981; Fahien, *et al.*, 1985; Fahien, *et al.*, 1988; Fahien, *et al.*, 1989; Teller, *et al.*, 1990). However, the association and dissociation of transiently formed hetero-enzyme complexes may modulate enzyme activity in "real time", since these complexes are dynamically influenced by changes in the concentration of a number of key metabolites

and the levels of enzymes used in these *in vitro* studies are significantly lower than their mitochondrial concentrations (Fahien and Kmiotek, 1983; Fahien, *et al.*, 1985; Fahien, *et al.*, 1989). AAT can form a complex with glutamate dehydrogenase (GDH) with high affinity (Fahien and Kmiotek, 1983). This interaction would assure that the α-ketoglutarate synthesized by GDH, could be transferred directly to the AAT with the net production of oxaloacetate and glutamate.

Under conditions of high levels of long-chain fatty acyl CoAs, it is known that GDH is inhibited (Kawaguchi & Bloch, 1976) and is possible that the GDH-AAT complex could be disassembled. Alternatively, α-ketoglutarate can be formed by amino acid transamination (by enzymes such as alanine, leucine and tyrosine transaminases), and palmitate has been reported to increase the affinity of AAT for α-ketoglutarate. This suggests that binding of palmitate could cause a conformational change in the AAT that facilitates binding of α-ketoglutarate, thus displacing the equilibrium of the reaction towards the synthesis of oxaloacetate and glutamate (Teller, *et al.*, 1990). The oxaloacetate formed by AAT could take part in the TCA cycle or gluconeogenesis. Under these conditions, the production of glutamate could arise from both inhibition of GDH and by activation of AAT. This prediction is consistent with the established fact that the liver has a net production of glutamate and glutamine during prolonged starvation (Cahill et al., 1981; Ishikawa 1976).

In general, we would expect all putative palmitoylated transaminases (e.g. alanine, leucine and tyrosine transaminases) to be activated by palmitoylation. This mechanism would assure availability of all or most of amino acids under conditions of nitrogen sparing.

6.3.2 Cross-talk between carbohydrate catabolism and fatty acids

Another enzyme detected to be fatty acylated using azido fatty acid analogues is pyruvate carboxylase [E.C. 6.4.1.1; PC]. PC is a member of the family of biotin-dependent carboxylases that catalyses the irreversible ATP-dependent carboxylation of pyruvate to form oxaloacetate which may be utilized in the synthesis of glucose, fat or some amino acids (Utter, *et al.*, 1975; Attwood and Keech, 1984).

Acetyl-CoA derived from long-chain fatty acid oxidation is an allosteric stimulator of the inactive PC monomer to an active tetramer (Utter, *et al.*, 1975; Attwood and Keech, 1984). Palmitoyl-CoA has also been described as an allosteric activator of PC activity, most likely acting at the same site as acetyl-CoA (Rohde, *et al.*, 1986).

Our results suggest that under conditions where fatty acids are elevated such as starvation, PC activity would be increased, and thus be involved in the maintenance of the serum glucose levels observed during prolonged starvation. Furthermore, if glucose levels are maintained and gluconeogenesis is increased, we would expect glycolysis to be reduced. In line with this idea, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was recently shown to

be palmitoylated and thus, inhibit GAPDH activity (Yang, *et al.*, 2005). GADPH plays a central role in glycolysis catalyzing the conversion of glyceraldehydes 3-phosphate to 1,3-biphosphoglycerate in a reaction accompanied by the reduction of NAD to NADH. This finding implicates a novel mechanism by which fatty acid metabolites can modulate glucose flux and induce insulin resistance.

6.3.3. Adjustments of metabolism to starvation

In the first 2 days, of a fast the liver glycogen quickly falls to about 10% of its normal concentration and then stays constant at this lower level for extended periods of fasting. The blood glucose level, however, remains relatively constant (Figure 6.2) mainly due to glycogenolysis and gluconeogenesis (derived mostly from amino acid catabolism) for the first week and due to gluconeogenesis after that. Once the supply of easily metabolized glycogen becomes exhausted, the rate of utilization of triacylglycerols from the fat deposits increases. The onset of accelerated triacylglycerol oxidation is accompanied by an increase in ketone-body production. The long-term maintenance of glucose levels is mainly achieved by amino acid catabolism and gluconeogenesis. Under these conditions, gluconeogenesis could be maintained by palmitoylation of PC (see section 6.3.2). Progressively, the use of amino acids for gluconeogenesis is reduced while the body relies more and more on fatty acid oxidation to derive metabolic energy. While the liver is flooded with fatty acids, they could inhibit amino acid catabolism at the urea

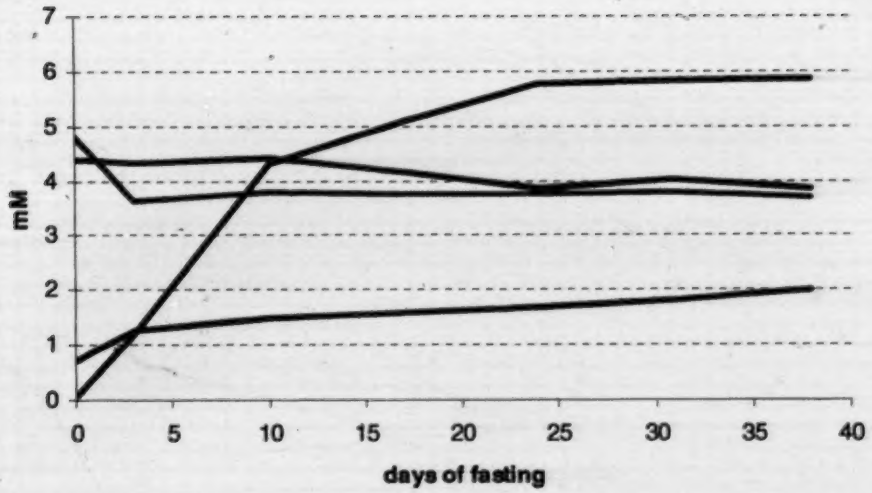


Figure 6.2 Main fuels in the blood during prolonged starvation.

Graph showing the serum concentration of the main fuels during prolonged starvation: free fatty acids (blue), β-hydroxybutyrate (black), α-amino nitrogen (red) and blood glucose (green). Adapted from Owen et al., 1968.

cycle level by inhibition of CPS 1 via its acylation as discussed in 6.3.1. Thus, the intermediate metabolites of the TCA cycle could be depleted since they are produced by amino acid catabolism. However, the TCA cycle cannot be shut down since it is required to work for the production of energy. This uncoupling of the urea cycle and TCA cycle could be achieved by activation of the AAT and possibly other transaminases. The activation of AAT would produce a net increment of oxaloacetate, an important intermediate in the TCA that would keep the cycle running. Furthermore, activation of transaminases would assure availability of amino acids under conditions of nitrogen sparing.

Overall, long-chain fatty acids would inhibit CPS 1 while activating AAT and PC. In this manner, long-chain fatty acids would assure nitrogen sparing (by inhibition of CPS 1), energy production (by activation of AAT and thus, allow the TCA cycle run although in a basal state) and maintenance of glucose levels (by activation of PC and therefore, gluconeogenesis) during prolonged starvation. Conceivably, similar metabolic adjustments would take place in physiological situations that involve high levels of fatty acids.

In Figure 6.3, the interactions between gluconeogenesis, the TCA and urea cycles under conditions of low and high long-chain fatty acids are illustrated.

6.4 Perspectives

The importance of understanding factors contributing to the regulation of key metabolic enzymes cannot be underestimated. Obesity is associated with

A

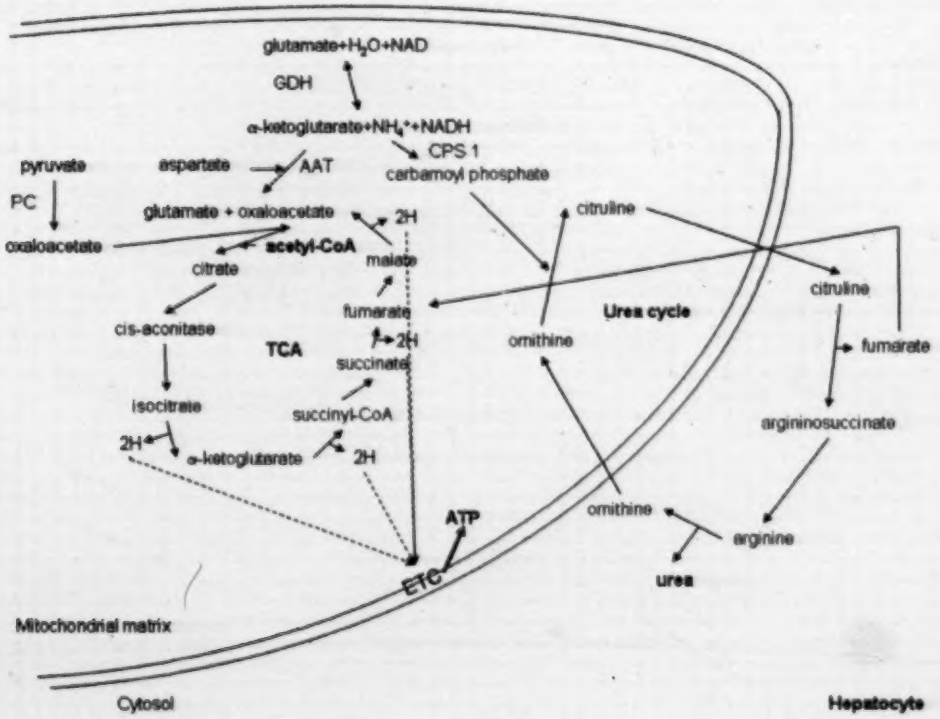


Figure 6.3 A. Interaction between TCA and urea cycles under conditions of low concentrations of long-chain fatty acids.

Diagram showing the interaction between gluconeogesis, TCA cycle and urea cycle under conditions of low (A) concentrations of long-chain fatty acids. In red we show the enzymes that have been detected to be fatty acylated in this thesis. Oxaloacetate is a crossroad between TCA and gluconeogenesis, while fumarate provides a link between TCA and urea cycles.

B

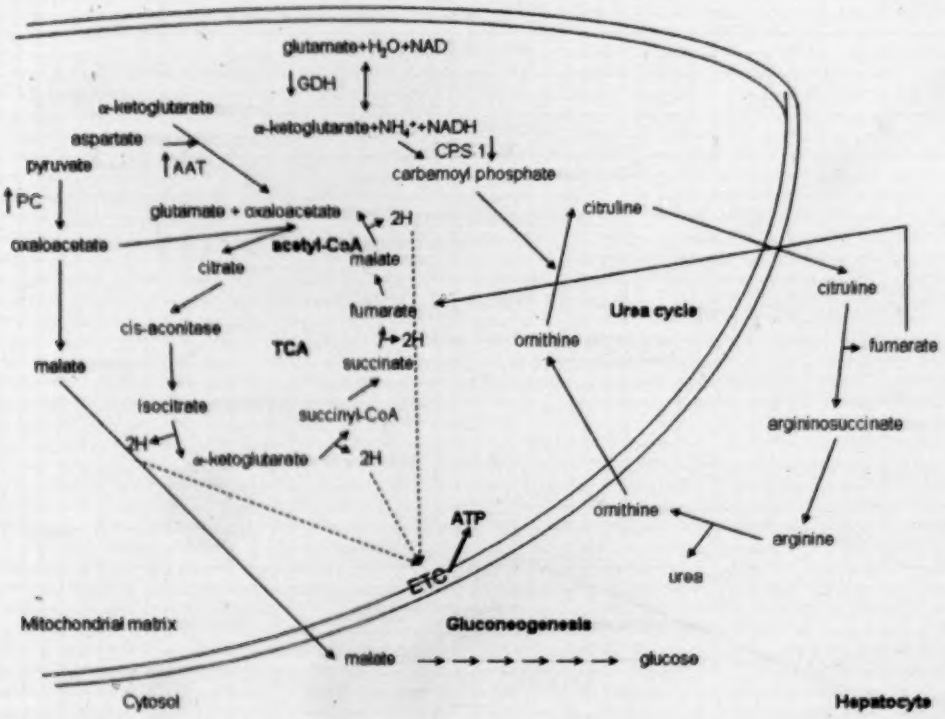


Figure 6.3 B. Interaction between gluconeogenesis, TCA and urea cycles under conditions of high concentrations of long-chain fatty acids.

Diagram showing the interaction between gluconeogesis, TCA cycle and urea cycle under conditions of high (B) concentrations of long-chain fatty acids. In red we show the enzymes that have been detected to be fatty acylated in this thesis. Oxaloacetate is a crossroad between TCA and gluconeogenesis, while fumarate provides a link between TCA and urea cycles. Red arrows next to enzymes indicte the predicted or known increase or decrease in activity in the presence of high concentrations of palmitoyl-CoA.

dislipidemia, insulin resistance, type II diabetes (Lewis, *et al.*, 1972; Richieri and Kleinfeld, 1995; Liu, *et al.*, 1998; Griffin, *et al.*, 1999; Segall, *et al.*, 1999; Shulman, 2000; Lewis, *et al.*, 2002), atherosclerosis and hypertension (Kleinfeld, *et al.*, 1996; Zhou, *et al.*, 2000; Chiu, *et al.*, 2001; Schaffer, 2002) which collectively constitute the metabolic syndrome (Moller and Kaufman, 2005; Smyth and Heron, 2006; Yach, *et al.*, 2006). The biochemical mechanisms underlying the metabolic syndrome are poorly understood.

One theory attempting to explain the metabolic syndrome postulates that the accumulation of fatty acids and their fatty acyl-CoA metabolites in excess of the cell's ability to appropriately metabolize them is actually toxic to cells and referred to as lipotoxicity (Unger, 1995; Lewis, *et al.*, 2002; Moller and Kaufman, 2005; Yang, *et al.*, 2005; Smyth and Heron, 2006; Yach, *et al.*, 2006). This theory is supported by the increased levels of intracellular fatty acyl-CoAs in patients with obesity and/or type II diabetes (Unger, 1995; Lewis, *et al.*, 2002; Moller and Kaufman, 2005; Yang, *et al.*, 2005; Smyth and Heron, 2006; Yach, *et al.*, 2006).Important advances in the field of mitochondrial fatty acylation would be provided by results that can be achieved in the short-term and that include:

1) Confirmation of the acylation of AAT and PC and characterization of the effect of palmitoylation on AAT and PC palmitoylation regarding activity, localization, and stability. This item is of utmost importance since these

enzymes link important pathways such as amino acid catabolism, fatty acids and gluconeogenesis.

2) Detection and identification of more mitochondrial fatty acylated proteins in order to obtain a possible consensus on the role of protein fatty acylation in mitochondria. To do so, the use of azido fatty acids and the Staudinger ligation would represent a tremendous advantage over the existing methodologies. Indeed our new protocol which showed to be more sensitive than the traditional radioactive and allows us to use a variety of labelings, would also allow the isolation of azido-modified proteins by pull down experiments or by affinity chromatography.

3) Since alkyl azides are not toxic to cells or animals, these analogues could be used in live animals for metabolic studies, including but not restricted to the study of diabetes and obesity.

All the above items as well as the data provided in this thesis are clinically relevant in the cases of long-chain acyl-CoA dehydrogenase deficiencies (Rocchiccioli, *et al.*, 1990), and especially in obesity (Lewis, *et al.*, 1972; Moller and Kaufman, 2005; Smyth and Heron, 2006; Yach, *et al.*, 2006), diabetes (Lewis, *et al.*, 1972; Richieri and Kleinfeld, 1995; Liu, *et al.*, 1998; Griffin, *et al.*, 1999; Segall, *et al.*, 1999; Shulman, 2000; Zhou, *et al.*, 2000) and obesity induced cardiomyopathies (Lardy and Shrago, 1990; Friedman and Leibel, 1992; Hill, *et al.*, 2003) where cellular acyl-CoA concentrations are established to be higher than normal (Lewis, *et al.*, 2002). This increase leads us to

consider that fatty acyl-CoAs regulate metabolic pathways by interacting with, or modifying the activities of key enzymes of the intermediary metabolism.

An in-depth knowledge of factors and mechanisms regulating metabolism can ultimately lead to new therapeutic strategies to overcome impaired metabolism.

CHAPTER 7

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